VASCULAR ACTION OF GLYCINE IN HYPERTENSIVE RAT MODELS

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By

Saswati Tripathy, MSc, MPhil

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S7N 5E5, Canada

OR

Dean

College of Graduate Studies and Research

University of Saskatchewan

107 Administration Place

Saskatoon, Saskatchewan S7N 5A2, Canada.

ABSTRACT

Glycine, a lipophilic non-essential amino acid biosynthesized from L-serine, L-alanine and L-threonine, plays a role in the biosynthesis of proteins, nucleotides, and glutathione. It is a neurotransmitter in the central nervous system and acts as a co-agonist at the N-methyl-D aspartate (NMDA) receptor. Oral administration of glycine is helpful in the management of schizophrenia. While long-term oral treatment with glycine is considered to exert a cardio-vascular protective role by overcoming endothelial dysfunction and oxidative stress, there are no systematic studies examining the cardiovascular effects of glycine. Recently, we showed that the precursor/metabolite of glycine, L-serine, evoked endothelium-dependent vasodilatation in rat mesenteric arterioles. Acute intravenous administration of L-serine produced a rapid, dose-dependent fall in blood pressure (BP) in both normotensive and hypertensive rats. These responses were abolished in the combined presence of the Ca^{2+} activated small and intermediate conductance K^{+} channel inhibitors, apamin and Tram-34/charybdotoxin. In contrast, intravenous administration of glycine evoked a fall in BP in normotensive Wistar-Kyoto (WKY) rats and an elevation of BP in spontaneously hypertensive rats (SHR), and in WKY rats subjected to chronic nitric oxide (NO) synthase (NOS) inhibition by oral treatment with NOS inhibitor, L-NAME (N^{G}-nitro-L-arginine-methylester). Therefore, *in vivo* and *in vitro* studies were designed to address the mechanisms that contribute to the opposite effects of glycine in normotensive *vs.* hypertensive rats.

Experiments were performed using 14 weeks old male WKY, chronic L-NAME treated WKY and SHR strains. *In vivo* studies involved examination of changes in systemic

hemodynamic parameters such as mean arterial pressure (MAP), heart rate (HR), total peripheral resistance (TPR) and cardiac output (CO) as well as regional hemodynamic parameters of changes in blood flow and vascular resistance in major organs/tissues following acute intravenous administration of glycine using fluorescent microsphere distribution technique. Parallel complementary *in vitro* studies were conducted to examine the effects of glycine on changes in basal tone and phenylephrine (PE) constricted tone in aortic rings with endothelium-intact and endothelium-denuded states after isolation from WKY and SHR strains. All these studies were conducted in the presence and absence of two NMDA antagonists, MK-801 and memantine.

In normotensive WKY rats, glycine (1 mmol/L) administration decreased MAP ($P<0.01$), TPR ($P<0.05$) while it increased CO ($P<0.01$) and blood flow to brain (215%), kidney (190%) and heart (160%). In SHR and L-NAME treated WKY rats, glycine administration elevated MAP and TPR but reduced CO ($P<0.01$) and blood flow to brain, kidney and heart. These effects were abolished in animals pretreated with either MK-801 or memantine. These data are consistent with the likely expression of vascular NMDA receptors activated by glycine in brain and kidney. Glycine (0.5-3.0 mmol/L) *per se* increased basal tone (E_{max}1.4g) in aortic rings from all rats and it was absent following incubation with NMDA antagonist(s). The concentration-dependent vasodilatation (I_{max}43%) evoked by glycine in PE-constricted rings with intact endothelium of WKY rats was attenuated by either MK-801 or L-NAME. Such vasodilator responses to glycine could be abolished by pretreatment with either NMDA antagonist or L-NAME. In contrast, in PE-constricted rings of SHR strain, addition of glycine enhanced the tone and this was abolished in the presence of NMDA antagonist, MK-801.

Taken together, these data suggest that NMDA receptors, likely present on both endothelium and vascular smooth muscle cells, predominantly in brain, kidney and coronary vascular beds as well as the conduit vessel, aorta, could contribute to the systemic and regional hemodynmic effects of glycine. In normotensive WKY rats, addition of glycine promotes endothelium/NO-dependent vasodilatation subsequent to Ca2+ mobilization evoked following endothelial NMDA receptor activation by glycine. Thus, glycine-induced dose-dependent, reversible fall in MAP and TPR in normotensive WKY rats is likely associated with NO-dependent/endothelium-mediated vasodilatation. However, in hypertensive rat models such as in the SHR model with endothelial dysfunction and reduced NO bioavailability or in chronic L-NAME treated rats, glycine administration promotes vascular NMDA receptor activation leading to enhanced vascular tone resulting in increased TPR and MAP.

These observations are important and critical in terms of considering glycine as a possible antihypertensive agent as this was proposed by several earlier reports that did not explore the direct vascular effects of glycine in hypertensive animal models.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AC:	adenylyl cyclase
ACE:	angiotensin 1-converting-enzyme
ACh:	acetylcholine
ADH:	antidiuretic hormone
ADP:	adenosine dihosphate
Ang I:	angiotensin I
Ang II:	angiotensin II
Ang:	angiotensin
ANP:	atrial natriuretic peptide
ARB:	angiotensin receptor blocker
AT_I	angiotensin receptor subtype I
ATP:	adenosine triphosphate
AVP:	arginine vasopressin
BF:	blood flow
BH_4:	tetrahydrobioptin
BK:	bradykinin
BP:	blood pressure
Ca^{2+}:	calcium
cAMP:	cyclic 3', 5' adenosine monophosphate
CBC:	canadian broadcasting corporation
Celloslove:	2-ethoxy-ethyl acetate
cGMP:	cyclic 3', 5' guanosine monophosphate

ChTX:	charybdotoxin
CNP:	c-natriuretic peptide
CNS:	central nervous system
CO:	cardiac output
CO_2:	Carbon dioxide
COX:	cyclooxygenase
CR:	concentration response curve
CVD	cardiovascular diseases
CVLM:	caudal ventrolateral medulla
DH-OLF	dihydroouabain like factor
DNA:	deoxyribonucleic acid
DOCA:	deoxycorticosterone acetate
DRI:	direct renin inhibitor
EC:	endothelial cells
EC_{50}:	half maximal effective concentration
EDHF:	endothelium-derived hyperpolarizing factor
EET:	epoxyeicosatrienoic acid
E_{max}:	maximal response
ENDO [-]:	endothelium denuded
ENDO [+]:	endothelium intact
eNOS:	endothelial nitric oxide synthase
ET:	endothelin
ET_1:	endothelin 1
F:	flow

FDA:	food and drug administration
F_i:	flow to individual sample
GABA:	gamma-aminobutyric acid
GCS:	glycine cleavage system
GHSR 1a:	growth hormone secretagogue receptor 1a
GLYT1:	glycine transporter 1
GSH:	glutathione
H_2O_2:	hydrogen peroxide
H_2S:	hydrogen sulfide
HR:	heart rate
I_i:	fluorescence intensity of the sample
$IK_{Ca:}$	intermediate conductance calcium activated potassium channels
iNOS:	inducible nitric oxide synthase
I_{ref}:	fluorescence intensity of the reference blood sample
JCC:	joint coordinating committee
JNC:	joint national committee
K_{Ca}:	calcium activated potassium
KCl:	potassium chloride
KOH:	potassium hydroxide
L-NAME:	N^G nitro L-arginine methyl ester
MAP:	mean arterial pressure
MEGJ:	myoendothelial gap junction
NAD^+:	nicotinamide adenine dinucleotide

NADPH:	nicotinamide adenine dinucleotide phosphate
NE:	norepinephrine
NHAES:	national health and examination survey
NHLBI:	national Heart, Lungs and Blood Institute
NIH:	national Institute of Health
nm:	nanometer
NMDA:	N-methyl D-aspartate
nNOS:	neuronal nitric oxide synthase
NO:	nitric oxide
NOS:	nitric oxide synthase
OLF:	oubain like factor
Pa:	arterial pressure
PAH	pulmonary hypertension
PE:	phenylephrine
PG:	prostaglandin
PGH_2:	prostaglandin H_2
PGI_2:	prostacyclin
PLA_2	phospolipase A_2
PLC:	phospholipase C
PNS:	parasympathetic nervous system
Pv:	venous pressure
PVR:	peripheral vascular resistance
r:	radius
R:	reference sample withdrawal rate (ml/min)

RAAS:	renin angiotensin aldosterone system
RNA:	ribonucleic acid
RVLM:	rostral ventrolateral medulla
SD:	Sprague-Dawley
SEM:	standard error of the mean
sGC:	soluble guanylate cyclase
SHMT:	serine hydroxymethyltransferase
SHR:	spontaneously hypertensive rat
$SK_{Ca:}$	small conductance calcium activated potassium channels
SNP:	sodium nitroprusside
SNS:	sympathetic nervous system
THF:	tetrahydrofolate
TNFα	tumor necrosis factor α
TPR:	total peripheral resistance
TXA_2:	thromboxane
VSMC:	vascular smooth muscle cell
WKY:	Wistar-Kyoto

LIST OF CHEMICALS

Acetylcholine

$CaCl_2$

Cellosolve acetate

Dextrose anhydrous

Fluorescent microspheres

Glycine

Heparin

KH_2PO_4

KOH

Memantine

$MgCl_2$

MK-801

NaCl

$NaHCO_3$

N^G-nitro-l-arginine-methyl ester

Phenylephrine

Potassium chloride

Sarcocine

Thiopental sodium

Tween® 20 and Tween®80

Chemicals were obtained from Sigma-Aldrich Canada Ltd, Invitrogen, Inc. (Eugene, U.S)

CHAPTER 1

INTRODUCTION

1.1. Hypertension as a global problem

High blood pressure (BP) also called hypertension, is a clinical condition with elevated arterial pressure (Kannel, 1990). Precisely BP is the force of pressure exerted by blood circulation against the arterial wall and is expressed in ratio of upper systolic and lower diastolic pressure in mmHg. Systolic pressure is the pressure under which the heart contracts and pumps out oxygenated blood to the system for metabolic need, whereas diastolic pressure is the pressure under which the heart relaxes and fills with blood. In hypertensive patients, the arterial wall becomes stiff due to lack of elasticity and the subsequent decrease in compliance increases resistance to blood flow in arteries. In this condition, the heart has to pump harder in order to push the blood through the small arteries. Increased BP eventually makes the heart weak and this leads to left ventricular hypertrophy and heart failure (Kannel, 1990 and 1996). This increase in BP is directly proportional to the resistance in arteries and inversely linked to lumen diameter and arterial wall thickening. Development of cardiovascular disease (CVD) state is related and resultant of high BP (Kannel, 1996). Sustained high BP increases the chances of cerebral stroke, due to inadequate blood flow and rupture of small arterioles in the brain leading to stroke (Levy et al., 1996).

Physiologically, BP is considered to be normal when the systolic and diastolic pressure value is 120/80 mmHg. However, several known and unknown factors are associated

with the elevation of these two values. According to the seventh report of Joint National Committee (JNC VII), systolic pressure ≥140 and diastolic pressure >90 mmHg is considered to be hypertension (Carretero et al., 2000; Chobanian et al., 2003). It has been reported that renal disease and diabetes contribute to high BP (Chobanian et al., 2003). A hypertensive state is established when pressure in the arteries remains high for an extended period of time. High BP is usually seen in the middle aged group and elderly people (Morrison et al., 1991). However, there are instances of some youngsters who develop high BP (Mouria et al., 2004). Once it develops, it has a tendency to become resistant to treatment and stays throughout. Life style changes, therapeutical intervention and regular pressure monitoring are required in most cases. Since the heart has to work under high pressure continuously, it eventually fails and this leads to target organ damage (Gregory et al., 2000). If hypertension is left untreated, it can lead to stroke, coronary heart disease and renal failure (Rossi et al., 1995). High BP affects the pumping ability of the heart, which in turn contribute adversely to the entire circulatory system. So it is essential and important to treat hypertension at its early onset.

Hypertension has become a serious global health problem and it is now recognized as one of the most common contributing factors to all forms of CVD which accounts for 20-50% of death (Castelli et al., 1984; Rosenfeld et al., 1995; World Health Organization (WHO) 1996). Although the exact cause of essential hypertension is still unknown, some of the major factors which permissively contribute to hypertension are diabetes, stress, obesity, lack of physical activity, smoking, high blood cholesterol, excess alcohol consumption, excess salt in diet (Elley et al., 2002; Klaus et al., 2009). Although there is a continuing

improvement in appropriate diagnosis and treatment of CVD, the most common cause for premature mortality and disability in adults is due to coronary artery disease and stroke (Hatmi et al., 2007; Coca et al., 2008). Due to an increasing risk of CVD all over the world irrespective of treatment, global risk assessment now has become very important next to therapy. Persons with hypertension are usually associated with dyslipidemia, impaired glucose tolerance and abdominal obesity (Manreque et al., 2010). Elevated blood pressure for all age groups is a major risk factor for atherosclerosis, coronary heart disease, heart failure, brain and myocardial infarction. Epidemiological studies have shown that factors associated with urbanization, environmental and geographical changes are related or correlated to the manifestation of hypertension and related cardiovascular diseases (Van et al., 2000). In most cases, pre or mild hypertension remains asymptomatic for long period of time and comes only under medical supervision when it manifests with severe complications. It has been reported that the rate of morbidity and mortality in patients with mild hypertension are such that a person aged 35 years with BP 140-150/90-100 mmHg without treatment, easily shortens life span by 15 years (Pickering et al., 1974). In such cases, treatment with antihypertensive drugs has shown that the overall incidence of complications can be significantly reduced and the death rate could be correspondingly minimized. Epidemiological studies show that in spite of a downward trend in mortality rate, hypertension still is the major cause of death in the western hemisphere (Waller et al., 1983; Stamler, 1985; Uemura et al., 1985; Tuomilehto et al., 1986; Ghannem et al., 1996). A recent report suggested that approximately one billion people worldwide have high BP, and by the end of 2025 this figure is expected to increase to one and half billion affecting one in three adults over the age of 20 (Kearney

et al., 2005). Globally around eighteen million deaths occur due to CVD, most of them are either suffering from diabetes or hypertension or both (Parvez et al., 2007). According to a recent assessment by the WHO, CVD stands as the number one cause of death globally. In 2004, approximately seventeen million people died from CVD, which represents 29% of the total global deaths. Seven million out of seventeen million deaths were due to coronary heart disease and six million were due to stroke (WHO, 2009). In developing and undeveloped countries 82% deaths are due to CVD. In the next two decades nearly twenty four million people will die mainly from stroke and heart related diseases (WHO, 2009).

North American prespective: However, the current survey demonstarted that there is an increasing trend in the prevalence of hypertension and CVD despite treatment (Kazuko, 2010). A report by National Health and Nutrition Examination Survey (NHANES) has found that hypertension, obesity and diabetes have increased in the United States in the past decade (Kazuko, 2010). Hypertension has become the leading cause for all forms of CVD in North America (Heart and Stroke Foundation of Canada, 2003). Hypertension is the leading global risk factor for mortality, and related heart and kidney diseases (Statistics Canada, 2005; Stone, 2010). According to a recent statistical survey report approximately five million adults, who accounts for nearly one fifth of the total Canadian population are suffering from hypertension (Stone, 2010). Apart from this population diagnosed many people remain unaware of their condition/undiagnosed due to lack of regular check up. Although 80% of the hypertensive population is under antihypertensive therapy, BP is being controlled only in 66% of that population (Stone, 2010). This study

shows that a small percentage of the population is resistant to current antihypertensive medications. Perhaps further diagnosis and more aggressive dose regimen would bring down the prevalence. BP still remains high in about one third of the hypertensive adults irrespective of antihypertensive therapy and regular BP monitoring. We need more research and studies to ensure total reduction of this fatal disease.

Canadian prespective: A recent Canadian Broadcasting Corporation (CBC) news bulletin highlighted that high BP is becoming a national crisis in younger Canadians (<20 years) due to increased rate of obesity, diabetes and physical inactivity (CBC News, 2010). According to the Heart and Stroke Foundation of Canada, there are more than 250,000 young Canadians with high BP (CBC News, 2010).

Saskatchewan Scenario: Another survey report described that CVD is the major cause of death in the senior population of Saskatchewan, which accounts for 26.2% of total death per year (A Health Profile of Saskatchewan Seniors, 2003). Taking all these issues into consideration, it is worthy and important to think and move forward in a constructive way to fight against high BP which is the key cause for all forms of CVD. Our ultimate goal is to live a disease free life; it is only possible if we are aware of ourselves. Awareness, and life style changes include physical exercise, dietary changes, weight loss and appropriate treatment with regular check up which would help us to achieve this desired goal.

1.1.1. Types of hypertension

Currently hypertension is classified into the three following categories:

1.1.1.1. Prehypertension

Previously, it was considered that systolic and diastolic pressure, which varies between 120-139 and 80-89 mmHg, is high normal. But this concept has been changed overtime. Recently the new guidelines provided by national Heart, Lungs and Blood Institute (NHLBI) which reclassified the upper systolic and lower diastolic pressure range for better monitoring and prevention of early onset of hypertension. In this new classification, they added a condition called prehypertension, along with primary and secondary hypertension. According to this new guideline, prehypertension defines systolic and diastolic pressure between 121-139 and 81-89 mmHg. This might be the early symptoms for and could lead to well established hypertension. Usually people diagnosed with prehypertension are advised to incorporate life style changes and are encouraged to check up BP at least once a year. People who fall under this prehypertensive category are normally prescribed non-pharmacological treatment that includes physical activity, diet control and minimize direct salt intake (NHLBI). According to the data provided by the NHAES III, since 1999-2000 approximately 31% of adult Americans were diagnosed as prehypertensive (prehypertension and borderline hypertension, 2007). The prevalence was more in male which is 39% compared to female 23% (Hasia et al., 2007). Based on a recent survey report by Durham Regional Hospital nearly 45 million American people fall under this category (Durham regional hospital, 2004). Table 1 describes the recommended blood pressure levels suggested by the American Heart Association.

BP Category	Systolic (mmHg)		Diastolic (mmHg)
Normal	120	and	80
Prehypertension	121 -139	or	81- 89
High-Stage I	140 -159	or	90 - 99
High-Stage II	≥ 160		≥ 100

Table 1. Classification of recommended blood pressure levels, American Heart Association. 2007.

Prehypertension is asymptomatic phase and has the potential to increase the risk of primary hypertension which subsequently could contribute to the development of CVD (Vanas et al., 2001; Qureshi et al., 2005). A person with prehypertension is three times more likely to have heart attack and 1.7 times to have heart related diseases compared to a normal individual (American Heart Association, 2005). So it has been suggested that prehypertension is the early phase of an impending well established hypertension (NHLBI report). Therfore it is very important to diagnose periodically and control prehypertension accordingly to overcome the risk of hypertension.

1.1.1.2. Essential or primary hypertension

Hypertension with no apparent cause is called essential or primary hypertension which accounts for 95% of the total hypertensive population (Carretero et al., 2000; Oparil et al., 2003; Hall et al., 2006; Hypertension: eMedicine Nephrology. 2009). Often essential or primary hypertension is also termed clinically as idiopathic hypertension.

Essential hypertension is a silent killer because it goes unnoticed without any visible complications initially. If not treated, at a later stage it contributes to cardiovascular complications like stroke, angina and heart failure. People with primary hypertension usually seen with combined elevation of both systolic and diastolic pressure. But in some cases mostly in elderly patients, only systolic pressure increases and is called as isolated systolic hypertension (Carretero et al., 2000). The specific cause for the combined systolic and diastolic or isolated systolic hypertension is not known. Although there is no specific reason mentioned yet how primary hypertension develops, several underlying factors may presumably be associated with it. A brief overview on this topic is provided below.

1.1.1.2.1. Factors associated with essential hypertension

Although there is no definite cause for the pathophysiological development of primary hypertension, some studies mention that genetic and environmental factors may contribute to primary hypertension (Morton et al., 1962; Avolio, 1995; Barlasinna et al., 1997; Rostand, 2003; Franks, 2008). Many genes are thought to be involved and work as a network system to bring about phenotypic changes, which are inherited from one generation to the other (Melander, 2001). The defective phenotypic changes are thought to be associated with abnormal cell membrane ion transport (Na^+, K^+ and Ca^{2+} channels present on the cell membrane), Na^+ excretion in the kidneys and sympathetic activation to promote the release of neurogenic hormones (Vikrant et al., 2001; Delpir et al., 2002; Hall, 2003; Ranke, 2003; Rosendroff, 2006; Eberhard et al., 2009). Some of the factors likely are associated with high BP are high dietary salt intake, smoking, heavy alcohol

consumption, ethnicity, impaired glucose tolerance, genetic disposition (family history of hypertension), age and gender. High dietary Na^+ intake has long been shown as a contributing factor in developing hypertension (Wyss, 2006; Theresa et al., 2006; Cook et al., 2007). However recent studies have shown that individuals taking low K^+, Mg^{2+} and Ca^{2+} in their diet are also likely to develop hypertension (Northcott et al., 2004; Nijenhuis et al., 2005).

To explain the onset of primary hypertension is not very easy but several hypotheses have been proposed to elucidate the issue. Briefly they are as follows:

(i) Malfunction in body's autoregulatory system.
(ii) Over stimulation of sympathetic nerve fibers supplying to heart and blood vessels
(iii) Inappropriate diuresis and natriuresis by the kidneys
(iv) Increase in blood volume and malfunction of Na^+/K^+ transport, which is regulated by hormones across the cell membrane of blood vessels and kidneys.

There are two important hypothesis proposed for the development of primary hypertension, the hemodynamic hypothesis and the neuronal hypothesis (Feig et al., 2003; De Luca et al., 2007).

The hemodynamic hypothesis is closely associated with the above mentioned hypothesis, whereas the neuronal hypothesis is specifically related to body's fluid volume and its regulation (Fetnat et al., 1997; Vasquez et al., 1997; Schrier et al., 1998; Feig et al., 2003;

De Luca et al., 2007; Spaan et al., 2009). Precisely fluid and electrolyte balance is interrelated. So we could safely say that both hemodynamic and neuronal hypotheses are closely related to each other and may be playing a role in developing hypertension.

1.1.1.3. Secondary hypertension

Hypertension with known cause is called as secondary hypertension. It is less common and accounts for only 5-10% of the total hypertensive population (Onusko et al., 2003). It is usually associated with other disease conditions like chronic renal disease, renal artery stenosis, pheochromocytoma, hypothyroidism, hyperthyroidism, chronic liver cirrhosis, hyperaldosteronism, iatrogenic or drug induced such as following long term corticosteroid therapy, pregnancy induced, sleep apnea and birth defect (Saken et al., 1979; Walker et al., 1994; O'Rorke et al., 2001; Wolk et al., 2003; Bradley, 2003; Seeley et al., 2007; Hulisz et al., 2008; Klabunde, 2009; Tanous et.al., 2009; Fallo et al., 2010).

1.2. Regulation of blood pressure

BP is regulated and controlled over a narrow range. It depends on the lumen size of the arterioles which contributes to change in the resistance of the vasculature. Basically BP is the function of three different properties called capacity, compliance and elasticity of the vascular compartments (Medical physiology, A.C. Guyton). Systems that regulate BP include nervous system, renin angiotensin-aldosterone system (RAAS) and humoral system. The major determinants of BP regulation are blood volume, cardiac output (CO) and total peripheral resistance (TPR). Blood volume in the vascular compartment depends largely on the rate at which blood flows in to and out of it. On the

other hand CO is the blood flow rate into the major arteries from the pumping action of the heart and determined mostly by venous return. Over a period of time CO and venous return has to be equal. They together represent the total blood flow in the circulatory system. Again venous return is detrmined by TPR and the pressure gradient for its return which depends on mean circulatory filling pressure which represents the capacitance function of the circulation. TPR represents the combined resistance in the circulation which is directly proportional to the rate at which blood leaves the arteries and arterioles. The relationship between BP, CO and TPR can be represented as BP=CO*TPR. Blood flow and resistance are two important determinants of the TPR. The fundamental principle of blood flow through different vascular beds should be equal to the difference between the arterial and venous pressure of vascular beds and this should be divided by resistance which determines the rate of flow. Resistance is always inversely proportional to the fourth power of the radius of the lumen of the vessel. Based on the above, the formula for the measurement of flow can be represented for calculation purposes as follows:

$F=Pa-Pv/R$ or $\Delta BF/r^4$. Where F = flow, Pa = arterial pressure, Pv = venous pressure, R = resistance, BF = blood flow, r = radius.

These determinants are continuously modified in an integrated and coordinated fashion by neural, renal and chemical control systems to maintain physiological homeostasis. This adjustment, controls efficiently and properly the entire perfusion system. But in case of pathophysiological conditions, these determinants are modified in an irregular manner which leads to cardiovascular complications. The entire BP regulatory system is

primarily controlled by two different mechanisms. The details of control mechanisms for BP regulation are described below.

1.2.1. Remote control system

The remote control system consists of parasympathetic nervous system (PNS), sympathetic nervous system (SNS) and the humoral system. They are important in short term control of BP by regulating BP and blood volume.

1.2.1.1. Nervous system

Blood vessels are primarily innervated by SNS where as heart is innervated by PNS and SNS.

1.2.1.1.1. Neural control

The baroreceptor reflex arch is an important component of neural control (Mc Corry, 2007). The second to second BP monitoring is controlled by arterial and cardiac baroreceptor reflex action in an integrated manner. These baroreceptors respond to vascular stretch. These actions are controlled by sending signals through afferent fibres from arterial and cardiac baroreceptors which transmits the signals through cranial nerves (IX and X) to nucleus of the tractus solitarius. The mediator is an excitatory neurotransmitter, glutamate, which activates caudal ventrolateral medulla (CVLM) in turn sends inhibitory fibres to rostral ventrolateral medulla (RVLM), the primary regulator of sympathetic nervous system. By inhibiting RVLM which sends excitatory glutaminergic fibers to sympathetic preganglionic neurons located in the spinal cord, this

inhibits sympathetic activation in response to increase in BP (Patron et al., 2009). This process controls BP dynamically by neural communication associated with baroreceptors which responds to the changing diameter of the blood vessels (Mc Corry, 2007, Patron et al., 2009). Neural control of BP regulation is a very complex process and occurs mostly by the reflex arcs, and to some extent involves chemoreceptors and higher brain centers (Zanzinzer., 1999, Dampney et al., 2005). The pathway of neural reflex arc and its response is somewhat integrated with vasomotor fibers, vasomotor center and pressoreceptors (Bernthal et al., 1944; Scherre, 1962; Miyakayo, 1988).

1.2.1.1.1.1. Vasomotor fibers

Vasomotor fibers are efferent nerve fibers from SNS innervating the membrane of vascular smooth muscle cells (VSMC) of arteries (Bonn, 1990). The vasomotor fibers release norepinephrine (NE) which is a potent vasoconstrictor (Westfall et al., 1985). Some of the vasomotor fibers also innervate the skeletal muscle and releases acetylcholine (ACh) which are known as vasodilator fibers (Welse et al., 1997). They play an important role locally in the blood flow to muscle during exercise (Blair et al., 1961; Joyner et al., 2007). However they do not have any control in regulating overall systemic circulation compared to vasomotor fibers that releases NE in VSMC of arteries (Kirkman et al., 2004; Thomas et al., 2004).

1.2.1.1.1.2. Vasomotor center

The vasomotor system controls BP and consists of a tangle of sympathetic nerve fibers located in the medulla (Lindsley, 1956; Engel et al., 1945; Cusingh et al., 1901; Shawn et al., 2004). Impulses are conducted from the vasomotor center through the

vasomotor fibers which are innervated to the arteries (Sergeeva, 1957). This impulse conduction depolarizes VSMC and results in an arteriolar contraction which we often call vasomotor tone. With the increase in impulse conduction, arteriolar contraction increases which contributes to the elevation of systemic BP (Hungerford et al., 2000; Welsh, 1994). With the subsequent reduction in firing there is a decrease in impulse conduction which decreases arteriolar contraction and we see a reduction in systemic BP (Gavaghan, 1998).

1.2.1.1.1.3. Pressoreceptors

Pressoreceptors are found in all large arteries of the thorax, neck, carotid and aortic sinuses, which can detect the changes in the arterial pressure (Jeweet, 1964; Scher, 1977). With the increase of arterial pressure these receptors stretch and send a fast impulse to the vasomotor center (Rodbard et al., 1951; Miyakawa, 1988; Gavaghan, 1998). Once the impulse reaches the vasomotor center, it reduces the impulse through the vasomotor fibers and brings down the BP by vasodilatation (Wang, 1953). It has been reported that afferent impulses from the pressoreceptors modulate the cardiac inhibitory center which decreases HR and cardiac contractility (Rothe et al., 1990). This in turn decreases mean arterial pressure (MAP) and activates lowpressure receptors, which ultimately disinhibit the RVLM and stimulate preganglionic sympathetic nerve fibers to release NE and constricts blood vessels with subsequent increase in BP to physiological level (Ludbrook J, 1983). The pressoreceptors are good at short term changes in BP (Ludbrook J, 1983; Collins R, 1990). However, there is no direct evidence as such for their role in regulating BP in chronic hypertensive state since threshold BP level for firing often changes or adapted in this disease condition (Yang et al., 1991).

1.2.1.1.1.4. Chemoreceptors

These receptors are mainly found in the aortic arch and the large arteries of neck (Timmer et al., 2003, Ishii et al., 1985). Although they contribute to the BP, their role is important during respiration (Ishii et al., 1986, Benchetrit et al., 1997). Primarily they sense a decrease in oxygen content and increase in hydrogen ion level in blood. By doing so they generate and send an impulse to the vasomotor center through the vasomotor fibers resulting an increase in vasoconstriction, led to increases in BP (Suleman et al., 2008).

1.2.1.1.1.5. Higher brain centers

Higher brain centers include hypothalamus and cortex (Hilton, 1983). They play a role in the regulation of BP during a situation like fight or flight by relaying the information to the medullary center (Hilton, 1983; Green et al., 2008). Since reflexes that control BP are present in the medullary center, after receiving the information from the higher brain centers, it acts accordingly and increases BP all of a sudden by releasing NE (Folkow, 1960).

1.2.1.2. Humoral system

The humoral system controls BP mainly by chemical control and renal regulation.

1.2.1.2.1. Chemical controls

There are some endogenously formed chemicals such as epinephrine, NE, arginine vasopressin and atrial natriuretic peptide (ANP), which can alter BP by acting

directly on vascular smooth muscle cell (VSMC) or on the vasomotor center (Flynn. 1996). Chemical control is important in short term regulation and maintenance of BP (Quan et al., 2006, Lohmeire, 2003).

1.2.1.2.1.1. Adrenal medulla hormones

During certain conditions like excitement or stress, cells in the adrenal medulla synthesize and secrete two catecholamines into circulation; they are epinephrine and NE (Jarry et al., 1995). After being released into the blood, they bind to the adrenergic receptors present on the target cells and exert their effect which is commonly called as fight and flight response (Vingerhoetes, 2002). By acting directly on heart, it increases the cardiac contractility, conduction velocity, automaticity and CO whereas it increases vascular contractility and periphearal vascular resistance by acting on blood vessels. Both epinephrine and NE increase BP by acting directly on cardiac tissue as well as the peripheral vasculature (Herrmann, 1982 and Buckwalter et al., 1997).

1.2.1.2.1.2. Atrial natriuretic peptide

ANP is a vasoactive substance released from the atria of the heart in response to high BP due to volume overload (Flynn, 1996). It is a powerful vasodilator responsible for reduction in BP by reducing blood volume (Flynn., 1996, de Los Angeles et al., 2000). It has potential to reduce cerebrospinal fluid formation and has a generalized vasodilatory effect in brain (Lida et al., 2001). Secretion and release of ANP is an autonomic function and it contributes to the short term BP control (Portaluppi et al.,

1993). It has a short half-life due to the buffering effect of its clearance receptor subtype C (Yohio et al., 2003; Frajewicki et al., 1997).

1.3.1.2.1.3. Antidiuretic hormone (ADH)

Arginine vasopressin (AVP) is an antidiuretic peptide hormone synthesized in the hypothalamus of most mammals. It is then transported to the posterior pituitary via axons and released following appropriate stimuli (Schrier et al., 1980). AVP exerts its effect primarily by acting on its receptors, V1 and V2 (Kjaer, 1994; Zingg., 1996; Caldwell et al., 2006; Aoyagi et al., 2009). These receptors are present in blood vessels and kidney. The major function of AVP is to regulate extracellular fluid volume by controlling renal handling of water (Forsling et al., 1988). AVP acts on V_2 receptors located on renal collecting ducts to increase water permeability and this mechanism is cAMP-dependent (Fernandes et al., 2002; Schrier et al., 2006). This subsequently leads to a decreased urine formation. This decrease in urine formation increases blood volume, CO and arterial pressure. In VSMC, AVP binds to its V_1 receptors causes vasoconstriction via IP3 signal transduction pathway (Robert et al., 2005). This in turn increases arterial pressure and results in a compensatory increase in peripheral vascular resistance. However, the main stimulus for AVP release is ‘hypovolemic shock’.

1.2.1.2.1.4. Ouabain like factor and dihydroouabain like factor

Ouabain like factors (OLF) and dihydroouabain like factor (DH-OLF) are endogenous cardinolides, synthesized by adrenal cortex and serves as a circulating hormone in the blood (El-Masri et al., 2002; Boulanger et al., 1993; Hamlyn et al., 1991).

They resemble digitalis glycoside in their structure and function (Goto et al., 1998). Like digitalis glycoside, OLF and DH-OLF inhibit Na^+/K^+ ATPase in the cell membrane. By inhibiting Na^+/K^+ ATPase, it increases intracellular Na^+ level and increases vascular tone (Goto et al., 1998). They induce natriuresis, thereby reducing plasma osmolality, and blood volume and BP in conditions of essential hypertension, but are also involved in the pathogenesis of essential hypertension (Bloch et al., 1988; Blaustein et al., 1993).

1.2.1.2.2. Renal regulation

The renal regulation plays an important role in long term BP control. It can be classified into two different but interrelated systems, the renin angiotensin and aldosterone system (RAAS) and the kallikrein kinin system (Vecsei et al., 1978; Matsuda., 1984; Nolly et al., 1992; Yayama et al., 2000).

1.2.1.2.2.1. Renin angiotensin and aldosterone system

RAAS is a hormone system responsible for long term control and regulation of both blood volume and BP (Garcia et al., 2003; Ganten et al., 2005). In response to decreased blood volume, BP, Na^+ and Cl^- delivery, the kidney releases a hormone called renin into the circulation (Garcia et al., 2003). Circulatory angiotensinogen released from liver is an alpha 2 globulin and acts as a substrate for renin. Angiotensin-I (Ang-I) is an inactive peptide primarily formed by the action of renin on angiotensinogen (Jackson et al., 1986; Jan-Danser, 1996). Ang I converting enzyme (ACE), a kinase, found predominantly in lung capillaries converts biologically inactive peptide Ang I to Ang II which is biologically active and a potent vasoconstrictor (Brosnihan, 2005). Ang-II

increases BP by activating AT_1 receptor present in the vasculature and is also responsible for the secretion of aldosterone from the medullary cortex (Williams, 1972; Brothers et al., 2006). Aldosterone is a Na^+ retaining hormone (Williams, 1972). Kidney tubules retain Na^+ and takes water with it which in turn increases reabsorption of Na^+ and water into the blood (Pratt, 1982). This process increases blood volume which increases BP due to excess Na^+ and plasma water retention (Sherwood, 2001).

Besides its role in longterm BP control through blood volume adjustment, RASS also intervenes with other regulatory systems. It plays an indirect role in baroreceptor reflex action and sympathetic nervous system function (Reid, 1992). Several reports suggest that Ang-II also acts centrally and interferes with the baroreflex control system. Normally, in response to high BP, baroreceptors activate and bring down the BP to the minimum firing threshold level but RAAS overrides this activity and compromises the baroreceptor mediated BP lowering effect (Reid., 1992; Max et al., 2002). During SNS activation, endogenous Ang-II plays an important role which increases the sympathetic out flow. Increased sympathetic out flow increases HR and BP (Rabbittes et al., 2009). RAAS has long been an important therapeutic target for lowering BP compared to other pathways. Currently, blockade of this pathway stands alone as a gold standard for reduction in preload and afterload which decreases BP, mediates substantial improvement in renal impairment, restores endothelial function and improves cardiovascular health (Yokokawa et al., 2008; Sever et al., 2009).

1.2.1.2.2.2. Kallikrein kinin system

The kallikrein kinin system is an important mediator of kinins that includes bradykinin and kallidin (Dendorfer et al., 1994). Both bradykinin and kallidin are

vasodilators (Santiago et al., 1995; Marshman et al., 1996). ACE inhibition is the front line approach to treat high BP. ACE inhibitors not only reduce Ang-II and subsequent aldosterone formation, but also increase the formation of bradykinin. Bradykinin is a vasodilator and plays a role in overcoming endothelial dysfunction by augmenting NO and prostacyclin (PGI_2) generation (Stanisavljevic et al., 2006). Kallidin or Lys-bradykinin is a decapeptide released from low molecular weight kininogen from the tissue kallikrein (Chagas et al., 1992). Both the kinins activate phospolipase A_2 (PLA_2) and facilitate the formation of prostaglandins (PGs) through arachidonic acid cascade (Warhurst et al., 1987). Most PGs are beneficial and modulate endothelial dysfunction, hence play a protective role against development of hypertension (Granger, 2009).

1.2.2. Local control system

Local control system regulates blood flow in the tissue or within the regional vascular beds (Shepherd et al., 1979; Lautt, 1981 and 1985; Monos et al., 1995). Local autoregulation of blood flow is crucial in order to maintain the vascular perfusion pressure (Lautt, 1981 and 1985). This system can be classified further into two subclasses, myogenic and the metabolic control.

1.2.2.1. Myogenic control

VSMC of arteries and arterioles respond to the local mediators to optimize perfusion which in turn contribute towards the systemic BP. Briefly, VSMC in blood vessels are directly affected by changes in BP. Blood flow to a vascular bed depends on pressure and resistance (Voelkel et al., 2000). If blood flow is low to a particular organ,

due to high resistance then adjacent arterioles normally relax to accommodate the flow. Conversely, if blood flow to an organ is very high, adjacent arterioles again contract, thus accommodating blood flow to appropriate level (Lautt, 1985). Most importantly, through myogenic control, tissues are somewhat self regulating and accommodating to changes in pressure, resistance and flow which is very important in terms of tissue perfusion and maintenance of systemic BP.

1.2.2.1.1. Endothelium derived vasodilators

Endothelium is the monolayer of cells that line the innermost layer of the blood vessels which releases several contractile and dilatory mediators. Vasodilatory factor and mediators released from the endothelium are NO, PGI_2, endothelium derived hyperpolarizing factors (EDHF) and carbon monoxide (Moncada et al., 1979; Furchgott et al., 1980; Maines et al., 1997; Christian et al., 1999; Brandes et al., 2000; Naik et al., 2002). Recently a report suggested that hydrogen sulfide (H_2S) has potential to hyperpolarize VSMC, thus H_2S can also be termed as an EDHF (Yang et al., 2009).

1.2.2.1.1.1. Nitric oxide

NO is the most important endothelium derived vasodilator and one of the most important signaling molecules which contribute to vascular homeostais by inhibiting platelet aggregation, vasoconstriction and leukocyte adhesion (Palmer et al., 1987). NO keeps endothelium nonthrombotic (Palmer et al., 1987). Many studies have shown that NO bioavailability is directly related to endothelial function (Sibal et al., 2009). Endothelial dysfunction associated with hypertension, diabetes and all forms of CVD

involves, a compromised or reduced state of NO bioavailability (Sibal et al., 2009; Kearney et al., 2008). NO is synthesized along with L-citruline from L-arginine by the action of nitric oxide synthase (NOS) in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobioptin (BH_4) and reduced molecular oxygen (Hibbs et al., 1987; Ignarro et al., 1989; Stanger et al., 1994). NO is responsible for the maintenance of vascular tone under normal physiological conditions (Palmer et al., 1987; Ignaro et al., 1989). Three different types of NOS have been identified. They are neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Both eNOS and nNOS are constitutively present in vascular endothelium and are Ca^{2+} dependent for their activity where as iNOS is expressed in response to inflammatory stimuli and does not require Ca^{2+} for its activation (Paulus et al., 1995). It has been reported that although eNOS is constitutively expressed, its expression can be altered or augmented by sheer stress, sex hormone, and exercise (Bayraktutan et al., 1998). In these conditions, eNOS gene expression increases several fold (Forstermann et al., 1998). On the otherhand hypoxia and increased generation of tumor necrosis factor α (TNF α) could down regulate mRNA and protein expression level for eNOS (Bayraktutan et al., 1998; Forstermann et al., 1998; Li et al., 2002; Anderson et al., 2004). eNOS is predominantly present in vascular endothelial cells (EC) and facilitates NO synthesis in the vascular endothelium (Paulus et al., 1995; Kobayashi et al., 2001; Li et al., 2006). Endothelium derived NO released into the vascular lumen diffuses to adjacent VSMC where it binds to the heme moiety of soluble guanylate cyclase (sGC) and increases the formation of cyclic guanosine monophosphate (cGMP). cGMP is responsible for phosphorylation of several cellular proteins resulting in VSMC relaxation and vasodilatation (Hardman et al., 1969;

Katsuki et al., 1997; Rapoport et al., 1983; Ignarro et al., 1989). NO counter balances the vasoconstrictor effect exerted by noradrenaline, serotonin, endothelin and Ang-II (Rubanyi et al., 1993). Hormones in pathological conditions, increase NO generation, contribute to enhanced oxidative stress and pathogenesis of atherosclerosis (Garg et al., 1989; Karya Y et al., 1989, Ross., 1993). Under physiological settings, NO plays a pivotal role in the regulation of BP by relaxing VSMC (Shesely et al., 1996). Several reports have demonstrated that eNOS gene knockout mice develop severe hypertension (Shesely et al., 1996; Mashimo et al., 1999; D'Souza et al., 2003)**.** Small arterioles isolated from eNOS gene knockout mice are resistant to ACh mediated relaxation (Huang et al., 1995; Chan et al., 2003). It has already been established that patients with essential hypertension have reduced levels of NO due to progressive endothelial dysfunction compared to normal individuals (Forte et al., 1997; Forte et al., 2000). Supplementation of exogenous NO, administration of NO donors or NO releasing compounds provide enormous beneficial effect by improving endothelial function in disease states such as hypertension and diabetes (Pearce et al., 2008; Carreiro et al., 2009). Chronic inhibition of NOS reduces NO bioavailability and increases peripheral vascular resistance which causes BP to increase (Stamler et al., 1994; Mikhail et al., 1997; Rees et al., 1990; Li et al., 2000).

1.2.2.1.1.2. Prostacyclin

PGI_2 is a member of the family eicosanoids. Prostacyclin is synthesized in endothelial cells from prostaglandin H_2 (PGH_2) by the action of enzyme prostacyclin synthase and exerts its action in a paracrine fashion (Tohgi et al., 1992). PGI_2 inhibits

platelet aggregation by inhibiting platelet activation and is a potent vasodilator. It binds to the platelet G-protein coupled receptor, activates adenylate cyclase and increases the level of cyclic adenosine monophosphate (cAMP). It binds to its receptors in the endothelium and increases cAMP by activating adenylate cyclase (AC). cAMP activates protein kinase A and phosphorylates several proteins which inhibits myosin light chain kinase and results in VSMC relaxation. An increased level of cAMP also inhibits platelet activation and inhibits intracellular Ca^{2+} level in VSMC (Moncada et al., 1979). PGI_2 is reported to be beneficial in the treatment of pulmonary hypertension (Murthy et al., 2010). In a normal physiological state, there is a dynamic balance maintained between endothelial derived contactile and dilatory factors. But, in cardiovascular disease state, it has been reported that bioavailabilty of NO and PGI_2 decreases substantially due to endothelial dysfunction which then contributes to the increased activity of contractile factors such as endothelin-1 (ET-1), thromoboxane A_2 (TXA_2) and Ang II (Endemann et al., 2004).

1.2.2.1.1.3. Endothelium derived hyperpolarizing factor

It has been proposed that EDHF is a vasodilatory mediator released by endothelial cells which leads to NO/PGI_2 independent vasodilatation, by hyperpolarizing adjacent VSMC (Garland et al., 1996). The mechanism of action and the nature of EDHF are controversial, since its effect is heterogenous and varies between species, strain and vascular beds (McGuire et al., 2001). Several endogenous agents are currently considered to serve as candidate molecule serving the EDHF function. They are: K^+, cytochrome P450 (CYP)-derived metabolite of arachidonic acid called

epoxyeicosatrienoic acid (EET), hydrogen peroxide (H_2O_2), and C-natriuretic peptide (CNP) (Dong et al., 1997; Edwards et al., 1998; Fleming et al., 2000; Matoba et al., 2003; Chauhan et al., 2003). Finally myoendothelial gap junction (MEGJ) has also been proposed to produce an EDHF like effect in many arteries (Griffith, 2004). All EDHF mediated responses are abolished in the combined presence of apamin (SK_{Ca} channel inhibitor), TRAM-34 (IK_{Ca} channel inhibitor), and charybdotoxin (IK_{Ca} and BK_{Ca} channel inhibitor) or ouabain (Na^+ pump inhibitor), Ba^{2+} (K_{ir} channel inhibitor) and depolarizing potassium (Quilley et al., 1997; Edwards et al., 1998).

1.2.2.1.2. Endothelium derived vasoconstrictor factors

Under normal physiological conditions, endothium derived vasodilatory and contractile mediators are in dynamic balance (Vanhoutte et al., 1986). In disease states such as diabetes, hypertension, and atherosclerosis, this balance is no longer maintained due to reduced bioavalability of NO (Griffith el al., 1988). Reduced NO production due to endothelial dysfunction augments enhanced vasoconstrictor activity by contractile mediators. This augmented activity of contractile mediators contributes exponentially to VSMC contraction which leads to high BP (Poulis et al., 2008).

1.2.2.1.2.1. Endothelin

ET was identified in 1988 and is abundant in most vascular tissues (Yanagisawa et al., 1988; Rubani et al., 1994; Schiffrin, 1999). It is released from endothelium, and a potent vasoconstrictor (Karwatowska et al., 1989; Schinelli et al., 2006). ET exerts its effect by acting on ET_A and ET_B receptors. ET exists in three different isoforms ET-1,

ET-2 and ET-3 (Arai et al., 1990; Sakurai et al., 1992; Hynynen et al., 2006). ET_A receptors are predominantly found in VSMC whereas ET_B receptors are predominant in endothelial cells (Batra et al., 1993; Hopfner et al., 1999). ET_A receptor has 100 fold more affinity for ET-1 compared to ET-3. ET_B receptor has equal affinity for all the three isoforms. ET_A receptor activation promotes vasoconstriction and contributes to high BP. ET_B receptor activation releases NO and decreases BP by relaxing VSMC. ET_B receptor has subtypes called ET_{B1} and ET_{B2}. ET_{B1} on the endothelial cells mediates vasodilatation and ET_{B2} receptors on the VSMC mediate vasoconstriction (Devenport et al., 1994). The ET_B receptor also serves as a "clearance receptor" for ET (Muramatsu et al., 1997; Kedzierski et al., 2001). Physiologically ET plays an important role in BP regulation, but in pathological conditions ET predominantly constricts blood vessels and contributes to vascular diseases (Noll et al., 1996; Benigni., 2000; Kedzierski et al., 2001). The ET antagonist bosentan is approved for treatment of pulmonary arterial hypertension (PAH). Sitaxentan sodium is currently under Food and Drug Administrations (FDA) review for the treatment of PAH (Dupuis et al., 2008; Abman, 2009). Bosentan was approved in Europe and submitted for, approval by FDA for the treatment of scleroderma such as ulcers in the fingers and toes (American Physiological Society, 2009).

1.2.2.1.2.2. Thromboxane A_2

Membrane phospholipids liberate arachidonic acid in response to physiological as well as pathological stimuli. Arachidonic acid down the road is converted to prostanoids by cyclooxygenase (COX) enzyme (Tohgi et al., 1992). Prostanoids are local in their action (Smith W L., 1992). Thromboxane A synthase is the enzyme that regulates the

synthesis of TXA_2 (Tohgi et al., 1992). Both TXA_2 and PGI_2 are degraded spontaneously under physiological conditions, whereas other PGs are enzymatically inactivated (Narumya et al., 2001). Although both PGI_2 and TXA_2 have opposite actions, they play an important role in the maintenance of vascular homeostasis. PGI_2 is produced by ECs, is a potent vasodilator and inhibits VSMC proliferation and platelet aggregation. Conversely, TXA_2 synthesis that occurs predominantly in VSMC binds to its thromboxane receptor and promotes platelet aggregation, vasoconstriction and VSMC proliferation. In conditions like hypertension, diabetes, atherosclerosis and vascular disease, the balance between PGI_2 and TXA_2 is altered due to progressive endothelial dysfunction. Less availability of PGI_2 and NO due to endothelial dysfunction exaggerates TXA_2 mediated vasocontractile effect, contributing to CVD (Abe et al., 1995; Ruschitzka et al., 1998).

1.2.2.1.2.3. Free radicals

Oxidative stress is one of the factors which contribute to vascular disease by generating free radicals. Increased level of free radical decreases superoxide dismutase (SOD), vitamin E and NO production by inhibiting NOS and PGI_2 synthase (Vijay et al., 1993; Vidal et al., 1998; Davidge et al., 2001). Free radical generation in turn is responsible for the production of superoxide anion and TXA_2 generation (de Artinano et al., 1999; Nakazono et al., 1999). Excess production of free radicals has been shown to increase vasoconstriction and VSMC proliferation, thereby contributing to pathophysiology of many diseases that include CVD, diabetes, artherosclorosis, and

rheumatoid arthritis (Giugliano et al., 1996; Aviram, 2000; Davi et el., 2005; Van et al., 2006).

1.2.2.2. Metabolic control

During metabolic activities, the vasculature produces different metabolites, such as carbon dioxide (CO_2), adenosine diphosphate (ADP), organic acids, K^+ and H^+ (Murrent et al., 2000). Increased metabolic activity increases the amount of metabolites that accumulate in the cells. They can exert a vasodilatory response by directly stimulating the arterioles to increase blood flow. This increase in blood flow decreases the accumulation of these metabolites. Subsequently cells try to contract and come back to its preactivation level (Murrent et al., 2000). Although VSMC contraction and relaxation depends on various dilatory and contractile mediators released from endothelial cells, increases in metabolic activity is one of the factors that also contribute to vasodilatation (Rong., 1998; Akata., 2007; Wong et al., 2009).

1.3. Management of hypertension

Our knowledge regarding the underlying mechanisms related to the pathphysiology of hypertension is limited. Currently, the overall approach to prevention, management by treatment with antihypertensive medications and appropriate patient care have been changed and improved. Over the years, new classes of pharmacological agents have also been introduced for the management of hypertension. Several new guidelines are proposed in the joint coordinating committee (JCC-7). JCC-7 and Canadian Hypertension Education Program (CHEP) guidelines provide current updates on

detection, evaluation, treatment and prevention of hypertension. In the management of hypertension, the following antihypertensives are in use; diuretics, β-blockers, α1 blockers, ACE inhibitors, angiotensin receptor blockers (ARBs) and calcium channel blockers. The renin inhibitor, aliskiren, is approved for the treatment of primary hypertension and has already shown promising results in clinical trials over other existing antihypertensives (Schmieder et al, 2009).

1.4. Vascular action of peptides

Many physiologically active peptides with effective antihypertensive properties have been discovered from different food proteins (Karaki et al., 1990; Yoshikawa et al., 2000). Some of them are suggested to have an ACE inhibition like property and are claimed to have potential in the treatment of hypertension (Fujita et al., 2000, Arai et al., 2001, Sun et al., 2009). Recently a peptide with amino acid sequence, Lys-val-Leu-Pro-val-Pro has been shown to decrease BP in hypertensive rats but the pharmacokinetic profile and mechanism of action is not that clear (Liu et al., 2007). There are some peptides derived from milk fermented by E. faecalis a bacterium with amino acid sequence LHLPLP, LHLPLPL, LVYPFPGPIPNSLPQNIPP, VLGPVRGPFP, and VRGPFPIIV have been shown to inhibit ACE and reduces BP in hypertensive rats (Miguel et al., 2006).

1.5. Vascular action of amino acids

Amino acids are building blocks of proteins and participate in metabolism. Amino acids are critical for the formation of coenzymes and serve as precursor for the synthesis of heme (Cox et al., 2005). They play an important role in nutrition (Young, 1994).

Several amino acids serve as excitatory as well as inhibitory neurotransmitters in the brain (Munro et al., 1986). Many reports have demonstrated that amino acids exert distinct vascular effect by modulating or activating membrane ion channels, receptors and transporters (Sgaragli et al., 1972). A role of amino acids in enhancing cardiovascular health has been demonstrated (Barinaga, 1990). Over a period of time several amino acids are suggested to play a cardiovascular protective role and lower BP. Briefly they are discussed below.

Taurine: Taurine is a naturally occurring organic acid. It is a major constituent of bile and is found in small intestine, in many mammalian tissues including human beings (Bouckenooghe et al., 2006; Brosnan et al., 2006). Taurine biosynthesis takes place in the pancreas via the cysteine sulphonic acid pathway. Taurine has beneficial effects in the proper maintenance of the skeletal system (Warskulat et al., 2004). It reduces fatty liver deposits and decreases liver cirrhosis in rats (Kerai et al., 1998). Oral supplementation of taurine in drinking water decreases BP in both spontaneously hypertensive rats (SHR) and in stroke prone SHR strains (Nara et al., 1978). Central administration of taurine (100 - 400 μg/kg) evokes a dose dependent fall in mean arterial pressure (MAP) in both anesthetized as well as conscious rats (Petty et al., 2002). Taurine and its potential cardiovascular health benefits have been demonstrated recently (Xu et al., 2008). Taurine is known to reduce ischemic disease and stroke (Yamori et al., 2009). Oral administration of taurine 6 g/day for a period of 7 days led to significant reduction in systemic BP in patients with essential hypertension (Militante et al., 2002). In rat and human studies, taurine mediated fall in MAP appears to be associated with reduction in overactive sympathetic nervous system (SNS) activity (Millitante et al., 2002; Hano et al., 2009).

The cardio protective effect of taurine is considered to act through multiple mechanisms (Millitante et al., 2002).

L-alanine: L-alanine, a non-essential amino acid, is found mostly in meat, egg, milk, beans, nuts and vegetables. It is a proteinogenic amino acid and nonpolar by nature. L-alanine administration decreases MAP in rodents (Garagli et al., 2002). L-alanine is reported to inhibit exploratory behaviour in SHR strain (Liljequist et al., 1982).

Tyrosine: Tyrosine is found in milk, meat, egg, banana and almonds. Dietary supplementation of tyrosine is reported to have the potential to decrease BP in hypertensive animals (Sveda et al., 1979).

Tryptophan: Tryptophan is an essential amino acid and is synthesized by plants and micro organisms from shikimic acid and anthranilate (Radwanski et al., 1995). It is a precursor for central neurotransmitter, 5-hydroxytryptamine/serotonin (Wolf et al., 1984). Some studies suggest that tryptophan and its analogs could decrease BP in normotensive and hypertensive rat models (Wolf et al., 1984).

L-arginine: Studies have shown that oral supplementation of L-arginine is highly effective in reversing endothelial dysfunction in several disease states (Rossitch et al., 1991). Oral or intravenous administration of L-arginine has been shown to be beneficial in preventing atherosclerosis, increases coronary blood flow in patients with heart disease and in alleviating intermittent claudication. L-arginine infusion lowers BP and prevents reclosing of arteries after balloon angioplasty (Maxwell et al., 1998). However, Schulman

et al have reported that, the addition of L-arginine to post infarction therapy did not show any improvement in vascular stiffness (Schulman et al., 2006). Instead, it may be associated with increased post infarction mortality. Thus, it shuld not be recommended to patients who have suffered acute myocardial infarction.

γ-amino butyric acid: γ-amino butyric acid (GABA) is an inhibitory neurotransmiter. Intra-cisternal infusion of GABA produces hypotensive effect in rodents (Brown et al., 1978).

Glutamic acid: Recently, a clinical trial has demonstrated that dietary supplementation with glutamic acid is associated with a significant BP lowering effect in hypertensive patients (Stamler et al., 2009).

L-serine: L-serine is a non-essential amino acid synthesized in the body from other metabolites. It is polar in nature and plays an important role in purine and pyrimidine synthesis, responsible for cell growth and survival. L-serine is conditionally essential in cell culture (McCoy et al., 1956). Oral supplementation with L-serine reduces methionine induced homocysteine formation along with a reduction in γ-glutamyl transpeptidase activity in humans (Verhoef et al., 2004). Close association of increased levels of plasma homocysteine and γ-glutamyl transpeptidase activity in metabolic syndrome and CVD has been demonstrated (Lee et al., 2007). In humans, oral supplementation of L-serine (>1.5 g/day) is found to be effective in the treatment of depression, schizophrenia, chronic fatigue syndrome and rare inborn errors of metabolism which are likely to be associated with L-serine deficiency. (Addington.et al., 1999; de Koning et al., 2003 and

2006). Our laboratory has shown that acute intravenous administration of L-serine evokes a dose dependent fall in MAP and it was more profound in two well established rat models of hypertension (Mishra et al., 2008a; Mishra et al., 2008b; Mishra et al., 2010). L-serine evoked vasodilatation is endothelium dependent but is not mediated through NO or PGI_2 (Mishra et al., 2007). Moreover, both the vasodilator and antihypertensive effect of L-serine is more profound in L-NAME treated rats. This vasodilator and antihypertensive effects of L-serine are blocked in the presence of SK_{Ca} and IK_{Ca} blockers, such as, apamin and charybdotoxin respectively. The decrease in MAP evoked by D-serine which is a metabolite of L-serine is much less in comparison to L-serine. In contrast, glycine which serves both as a precursor/metabolite to L-serine, evoked a decrease in the MAP in normotensive rats and an increase in the MAP in hypertensive rats in a dose dependent manner (Mishra et al., 2009). L-serine can be derived from or give rise to glycine. Although both L-serine and glycine decrease MAP in normotensive animals their magnitude of relaxation and sensitivity are different. Unlike L-serine, glycine mediated responses are sensitive to NMDA antagonists, such as, MK-801 and memantine. Interestingly, L-serine evokes a profound fall in MAP where as glycine evokes an elevation in MAP in hypertensive rat models (Mishra et al., 2008b).

These studies provide the rationale for establishing *in vitro* and *in vivo* studies for the direct vascular effects of glycine which is different from L-serine in hypertensive rats.

1.6. Glycine

Glycine one of the 20 naturally occurring amino acids that was discovered by the French Pharmacist, Henery Bracconut in 1820. The name glycine came from a Greek

word that translates as“for sugar”. Glycine is a sweet tasting, non essential amino acid, derived from other amino acids in the body such as L-serine and threonine. It is lipophilic in nature with a molecular weight 75.07 dalton and molecular formula $C_2H_5NO_2$. It is required for the systhesis of deoxyribonucleic acid (DNA)and ribonucleic acid (RNA), participates in the synthesis of bile acids, adenosine triphosphate (ATP), porphyrins, hemoglobin, peptides, proteins, creatine, glycogen, glutathione and L-serine in the body. It is conditionally essential in the systhesis of purines, heme and glutathione (Cox et al., 2005). The plasma concentration of glycine is 227.5 µmol/L in human and in rat the plasma concentration of glycine is 272 ± 25 (Polge et al., 1997; Palou et al., 1977; Christensen et al., 1946; Bonnet et al., 2007). Glycine serves as an inhibitory neurotransmitter in the CNS and acts as a co-agonist at N-methyl D-aspartate (NMDA) glutaminergic receptor. Glycine has the potential to increase growth hormone and is involved in the proper functioning of immune system (Kasai et al., 1978 and 1980). It plays an important role in the repair of damaged tissue and promotes tissue healing (Harvey et al., 1985). Glycine in combination with alanine and glutamic acid has been shown to have beneficial effect in reducing the prostate size (Feinblatt et al., 1958; Dumrau et al., 1962; Osiecki et al., 2004). Although glycine is not essential in the diet, it serves as an important micronutrient for the proper functioning of the body (Osiecki et al., 2004).

1.6.1. Sources of glycine

Glycine is the most abundant amino acid found in the body. It is present in all cells of the body. A large amount of glycine is found in the skin, connective tissue and in

the prostate cells (Osiecki et al., 2004). Glycine is plentily available in protein rich foods such as meat, fish and milk products (Osiecki et al., 2004). Glycine serves as a precursor to L-serine and under certain conditions; L-serine is also metabolized to glycine in the presence of serine hydroxyl methyltransferase (SHMT) and tetrahydrofolate (THF). Thus, L-serine and glycine are interconvertible depending on tissue availability of SHMT and THF. Table 2 lists the amount of glycine in milligram present in some of the dietary protein products.

Food	Amount	Glycine (mg)
1/2 Turkey breast - no skin	306 gm	4562
1/2 Chicken - no skin	329 gm	3458
Tuna, canned in oil	1 can (171 gm)	2391
Lean round beef, 0% fat	100 gm	2199
Chicken breast, no skin, roasted	140 gm	2133
Atlantic cod, cooked	1 fillet (184 gm)	1973
Lean veal leg, cooked	100 gm	1886
King mackerel, cooked	½ fillet (154 gm)	1922
Mozzarella, part skim	100 gm	4644

Table 2. Amount of glycine (mg) in dietary protein products.

1.6.2. Biosynthesis of Glycine

Glycine is a non essential amino acid that is derived from other amino acids such as L-alanine, L-serine and L-threonine in the body (Figure 1). In most living organisms SHMT catalyses this conversion in presence of a cofactor called pyridoxal phosphate (Nelson et al., 2005). Glycine also can be biosynthesized from isolated chloroplasts (Shah et al., 2001). Vertebrate liver produces an enzyme called glycine synthase participates in the glycine cleavage system (GCS) which catalyzes this synthesis but this process is reversible (Cox et al., 2005).

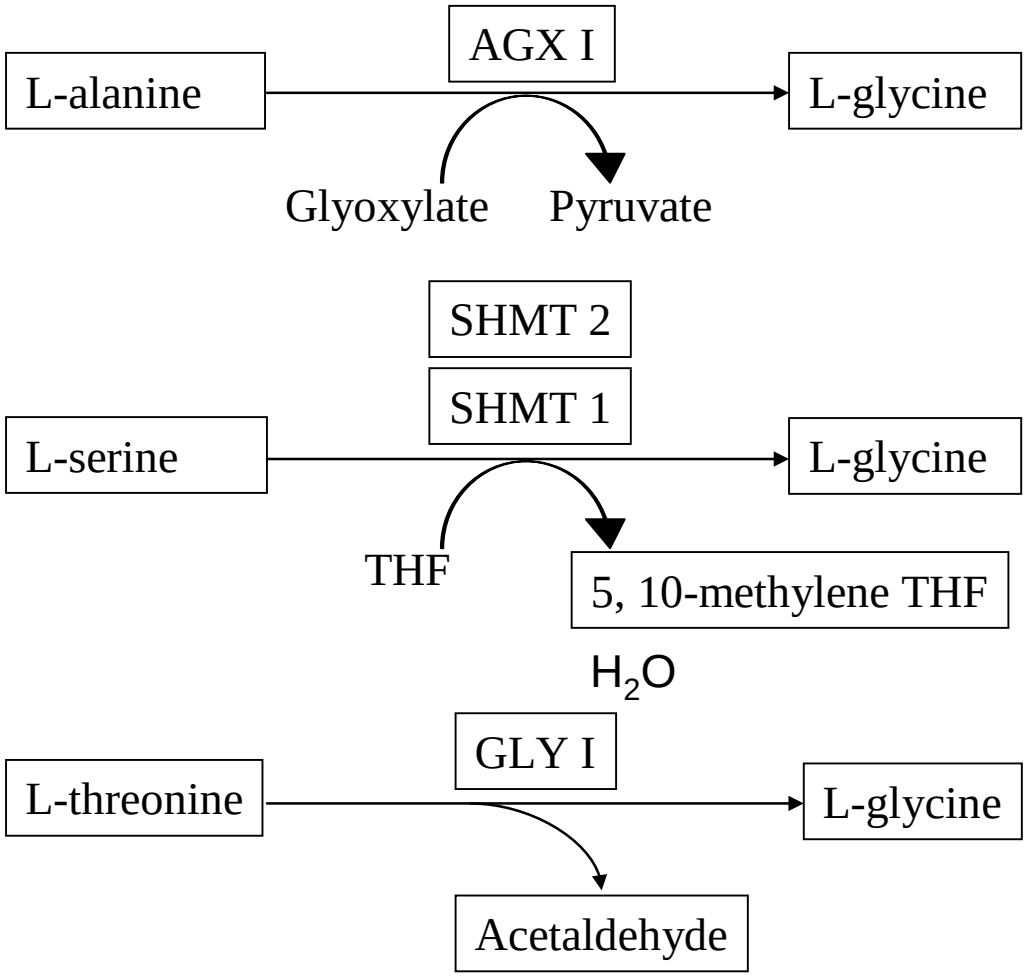

Figure 1. Schematic representation of glycine biosynthesis from L-alanine, L-serine and L-threonine.

AGX1, Alanine glyoxylate aminotransferase 1; GLY, Glycine; SHMT, serine hydroxyl methyltransferase; H_2O, water; THF, tetrahydrofolate;

1.6.3. Glycine and its metabolism

Glycine metabolism occurs through three different pathways (Cox et al., 2005). The most important pathway is catalysis of glycine by glycine synthase or glycine cleavage enzyme. The second pathway involves an enzyme called serine hydroxymethyltransferase (SHMT) that converts glycine to L-serine and subsequently L-serine is converted to pyruvate in the presense of another enzyme called serine dehydrase. The third pathway involves the conversion of glycine to glyoxylate by D-amino acid oxidase. Glyoxylate is then oxidised to oxalate in the presence of an enzyme called hepatic lactate dehydrogenase. This reaction requires nicotinamide adenine dinucleotide (NAD^+) to produce oxalate (Cox et al., 2005). Thus, glycine and L-serine metabolism are linked to glycolytic pathway of glucose metabolism.

1.6.4. Glycine and its receptor

Glycine is lipophilic in nature and can not be ionized since it does not exhibit chirality; moreover its R-group is a single hydrogen atom (Kleuss et al., 2003; Biochemistry of Amino Acids, 2009). Gylcine can easily cross the blood brain barrier (BBB) and exerts its central effect (Danysz et al., 1998). Primarily, glycine acts on its receptors that includes both the receptor subtypes and mediate its response (Biochemistry of Amino Acids, 2009). The first glycine receptor identified was a strychnine-sensitive one, found mainly in the spinal cord and lower brain stem (Cimino et al., 1996; Danysz et al., 1998; Jiang et al., 2004; Lynch, 2004). The other one is strychnine-insensitive and found in different areas of brain in the following order, hippocampus > cerebral cortex > caudate putamen > thalamus > cerebellum (Malosia et al., 1991). Glycine is considered

to be an inhibitory neurotransmitter in the brain and spinal cord (Lynch, 2004; Schmidt et al., 2006). Glycine receptors are coupled with inhibitory neurotransmitter gated Cl^- channels localized in the presynaptic membranes (Ehrlich et al., 1999; Turecek et al., 2001; Lynch, 2004). Glycine receptor has two subtypes, glycine-A and glycine-B (Danysz et al., 1998). Glycine-A subtype consists of α and β subunits. The α subunit is again subdivided into α_1, α_2, α_3 and α_4 isoforms (Malosia et al., 1991; Lynch, 2004). The function of the single β subunit is to anchor glycine receptors to the subsynaptic cytoskeleton with the help of cytoplasmic protein known as gephyrin (Lynch, 2004). Glycine-B subtype on the other hand is a strychnine insensitive receptor, known to serve as coagonist at glycine binding site of NMDA glutaminergic receptor (Danysz et al., 1998). The classical action of glycine by acting on its presynaptic glycine-A receptors that includes all isoforms (α_{1-4}) triggers normally a very weak depolarizing Ca^{2+} current due to Cl^- influx. This weak Ca^{2+} current not only helps in releasing the neurotramsmitter but also elicits an inhibitory post synaptic action potential (Turecek et al., 2001). Unlike the classical inhibitory action mediated by glycine by acting on its subtype glycine-A receptor, glycine is also required for activation of the NMDA receptor along with glutamic acid to cause influx Ca^{2+} (Danysz et al., 1998; de Koning et al., 2003).

1.6.5. Vascular action of glycine

Glycine is known to serve as an inhibitory neurotransmitter in brain and spinal cord. Glycine primarily opens chloride ion channels by activating its receptor. Chloride makes neuronal cell membranes more negative, and hyperpolarizes the neuron. This hyperpolarization exerts an inhibitory potential (Lynch, 2004). Glycine is a coagonist at

NMDA glutaminergic receptor along with NMDA and D-serine. Glycine prevents apoptosis in endothelial cells (Zhang et al., 2000). Glycine intake has been shown to decrease adipose cell size, plasma free fatty acid and BP in sucrose-fed rats (Hafidi et al., 2004). This BP lowering effect of glycine is suggested to be associated with a reduction in oxidative stress (Hafidi et al., 2006). Dietary supplementation with glycine has the potential to reverse the elevated systolic BP encountered in pregnant rats on a low protein diet (Jackson et al., 2002). Glycine regulates the production of pro-inflammatory cytokines in lean and mono sodium glutamate-treated obese mice (Aguilar et al., 2008). Glycine is reported to improve microcirculation and minimize reperfusion injury in rat liver (Zhong et al., 1996). Recently a report suggested that glycine protects human intestinal epithelial cells from oxidative damage (Howard et al., 2009). Glycine participates in synthesis of glutathione and this protective effect of glycine is probably due to restoration of glutathione. Another study suggested that glycine mediated cytoprotection in kidney and liver is independent of glutathione (Weinberg et al., 1992; Dickson et al., 1992). Another report suggests that glycine decreases oxidative stress induced by activation of inflammatory mediator, interleukin-2 (IL-2) (Katayama et al., 2007). Dietary glycine protects against colitis induced intestinal injury in rat models (Tsune et al., 2003). It has been shown that glycine transporter 1 (GLYT1) in gut epithelial cells protect the gut from inflammation (Mc Cole., 2010). Glycine intake may be beneficial in the treatment of inflammatory bowel syndrome. Glycine and its protective effect are extensively reported in the literature, but the effect of glycine administration in the management of CVD has not been addressed so far. Recently, we reported for the first time that L-serine decreases MAP in normotensive rats and this

effect is more pronounced in hypertensive rat models. In contrast administration of glycine, a precursor/metabolite of L-serine, decreased MAP in normotensive rats, but increased MAP in hypertensive rat models (Mishra et al., 2008a; Mishra et al., 2008b). The L-serine mediated depressor response is associated with a decrease in splanchnic vascular resistance in hypertensive rat models (Mishra et al., 2010). These studies provide impetus to look at the specific regional vascular beds, which may be contributing to this pressor response evoked by glycine in hypertensive rat models.

1.6.6. Glycine and its possible therapeutic uses

Glycine (2g/day) has been used along with other neuroleptics as add on therapy in the treatment of schizophrenia (Buchanan et al., 2007). Trimethyl glycine has been reported to have potential in the treatment of steatohepatitis (Miglio et al., 2000; Angulo et al., 2001; Abdelmalek et al., 2009). Long term glycine intake protects against oxidative stress. This suggests that its administration may be considered in the treatment of hypertension (Hafidi et al., 2006). A double blind study suggested glycine intake is useful in blood glucose regulation (Martinez-Abundis et al., 2003). Anhydrous trimethyl glycine is approved by FDA in the treatment of homocystinuria (Holm et al., 2005). Glycine being an inhibitory neurotrasmitter has potential in the management of neurologic disorders, hyperactivity, seizure, depression and bipolar disorders (manic phase) (Danysz et al., 1998; Hashimoto et al., 2006; Buchanan et al., 2007).

CHAPTER 2. HYPOTHESIS AND RATIONALE

2.1. Background and rationale for the present study

Our laboratory has primarily engaged in establishing the cardiovascular effect of novel peptides like ghrelin and segetalins. Ghrelin is a peptide hormone consisting of single chain of 28 amino acids and was discovered in 1999 (Kojima et al., 1999). It is released from the stomach and it is involved in growth hormone secretion by activation of ghrelin receptor GHSR1a/ GRLNR, a cell surface G protein coupled receptor that enhances phospholipase C (PLC) activation and elevation of cytosolic free calcium (Kojima et al., 1999; Wren et al., 2000; Cunha et al., 2002). Several laboratories have shown that, besides its classical physiological actions of increasing growth hormone release and decreasing food intake, exogenous administration of ghrelin evokes vasodilatation and a fall in MAP. It improves NO bioavailability and normalizing the imbalance between ET-1/NO within the vasculature of individuals with metabolic syndrome (Shinde et al., 2005; Manfredi et al., 2010). Our laboratory has demonstrated that the vasodilator responses of ghrelin in the perfused mesenteric vascular bed in rat models are not due to activation of ghrelin receptor, GHSR1a. Even des-octanoyl (des-acyl) ghrelin, which is a precursor and a metabolite of ghrelin, but not a ligand of GHSR1a, produced similar type of vasodilator response as ghrelin. These data suggested that the vasodilator response evoked by GRLN and des-acyl GRLN is not due to activation of GHSR1a (Moazed et al., 2009). Interestingly, several N-terminal peptide fragments of des-acyl GRLN also evoked a similar vasodilator response to des-acyl GRLN when endothelium was intact (Moazed et al., 2009). Serine residues are abundant

in the N-terminal sequence of des-acyl ghrelin (Kojima et al., 2001; Matsumoto et al., 2001). Also studies from different laboratories demonstrated that N-arachidonyl L-serine evoked vasodilatation in rat mesenteric arteries and in abdominal aorta when endothelium is intact (Milman et al., 2006). Thus, it is likely that the vasodilatation and fall in MAP evoked by ghrelin and its analogs could be due to serine residues present on the N-terminal sequence of ghrelin. This prompted us to undertake detailed studies to investigate the vascular responses to L-serine. *In vitro* addition of L-serine evoked endothelium-dependent but NO or PGI_2-independent vasodilatation (Mishra et al., 2008a). Moreover, both *in vitro* (vasodilator response) and *in vivo* (the fall in MAP) studies established that the responses to L-serine were significantly higher in rats with elevated BP following NOS inhibition by four days of oral treatment with L-NAME (0.7 mg/ml in drinking water given ad libitum). L-serine-induced concentration-dependent vasodilatation in the third order branches of rat mesenteric arterioles were abolished either following endothelial denudation or in the combined presence of SK_{Ca} and IK_{Ca} inhibitors, apamin and charybdotoxin/TRAM-34, respectively. Similarly, the dose-dependent fall in MAP evoked by L-serine was abolished in rats that were pretreated with apamin and charybdotoxin (Mishra et al., 2008a and 2008b). Like L-serine, D-serine also evokes a dose dependent fall in MAP. This decrease in MAP is much less in comparison to L-serine. In contrast, glycine which serves both as a precursor/metabolite of L-serine evokes a decrease in MAP in normotensive rats and an increase in MAP in hypertensive rats (Mishra et. al., 2008b). The effects of both L-serine and glycine were clearly dose-dependent. While a number of studies have suggested that glycine is cardiovascular protective, paradoxically, in both SHR and L-NAME treated hypertensive rats we have

reported that acute intravenous administration of glycine evokes increases in MAP (Mishra et al., 2008b). While apamin+charybdotoxin treatment abolished the fall in MAP evoked by L-serine, the responses to glycine (fall in MAP in normotensive rats and increase in MAP in hypertensive rat models) remained unaffected in rats that were subjected to combined pretreatment with apamin+charybdotoxin. These data confirmed that the altered responses to glycine (fall in MAP in normotensive rats vs. elevation in MAP in hypertensive rat models) are not mediated by activation of EDHF since responses were sensitive to inhibition by apamin+charybdotoxin/TRAM-34 (SK_{Ca} and IK_{Ca} inhibitors). However, pretreatment with the NMDA antagonist, MK-801, abolished glycine mediated responses (Mishra et al., 2008b). The mechanism associated with the opposing effects of glycine in normotensive vs. hypertensive rat models has not been fully elucidated and it is the intent of the present work to establish the mechanism. Moreover, the regional vascular beds that respond to glycine have not been established. A subsequent study from our laboratory has shown that the systemic hemodynamic responses of a profound fall in MAP evoked by L-serine in hypertensive rat models is mainly due to a decrease in splanchnic vascular resistance in L-NAME treated hypertensive and SHR models (Mishra et.al., 2010). It is unclear whether glycine evoked changes occur in the same regional vascular beds. This remains to be tested and it is the major focus of the present study.

2.1.1. Rationale for the regional hemodynamic study

Several studies using dietary glycine in diverse animal models and circumstantial evidence from human studies have indicated that glycine has potential to improve

endothelial function by reducing oxidative stress which subsequently contributes to the antihypertensive effect (Talman et al., 1989; Jackson et al., 2002; Yin et al., 2002; Hafidi et al., 2004; Hafidi et al., 2006). In support of these observations, a number of studies have also pointed out that glycine induces renal vasodilatation perhaps by activating glutamate NMDA receptors (Deng et al., 2002). NMDA receptor activation increases Ca^{2+} influx. If this exists in vascular ECs, glycine would increase generation of vasodilator mediators such as NO (following Ca^{2+} induced activation of NO, PGI_2 via PLA_2 activation and EDHF via activation of SK_{Ca}/ IK_{Ca}). On the other hand, NMDA receptor activation in VSMC could result in vasoconstriction and a concomitant increase in systemic vascular resistance. The presence of NMDA receptors has been shown in aortic smooth muscle cells (Crespi et al., 2000). Some reports suggested that dietary intake of glycine reduces BP (Jackson et al., 2002)**.** On the other hand our work has shown that, L-serine mediated vasodilatory and hypotensive effect was not shared by glycine since combination of SK_{Ca} and IK_{Ca} inhibitors abolished L-serine mediated effect but not glycine mediated response. However, a selective NMDA antagonist MK-801 abolished glycine mediated effect but not L-serine effect. Clearly L-serine and glycine evokes their vascular effect in normotensive and hypertensive rat models via different mechanisms, although glycine serves as a precursor and metabolite of L-serine (Mishra et al., 2008b). We have already shown in another study that L-serine evoked hypotensive effect is due to decrease in splanchnic vascular resistance (Mishra et al., 2010). Therefore, it is now important to explore the systemic and peripheral hemodynamic effect of glycine in normotensive as well as hypertensive rat models to establish which vascular beds are sensitive to glycine.

2.1.2. Rationale for the *in vivo* studies

In addition to MK-801, another NMDA receptor antagonist memantine (another open channel blocker) could be used to confirm glycine induced systemic hemodynamic effects are indeed mediated by vascular NMDA receptor activation. The overall effect of glycine on rat vascular NMDA receptors are minimal since much of glycine is transported by amino acid transporter into the cells and when this transport is blocked by employing a glycine transporter inhibitor, N-methyl glycine, sarcosine, as expected extracellular glycine levels would increase and this could result in a more profound effect of glycine on vascular NMDA receptors to promote Ca^{2+} influx in both normotensive and hypertensive rats.

2.1.3. Rationale for the *in vitro* studies

While these studies could provide pharmacological evidence from functional type studies for the recruitment of vascular NMDA receptors, these *in vivo* type experiments alone do not provide a mechanistic explanation for the fall in MAP in normotensive vs. elevation in MAP in hypertensive rats although both of these effects of glycine could be attenuated by more than one NMDA antagonists. To address this issue, *in vitro* type experiments will be performed using isolated ring preparations of rat aorta. The existence of NMDA receptors on rat aortic smooth muscle cells/rat aortic tissue has been previously demonstrated (Qureshi et al., 2005). However, the effects of glycine has not been investigated using aortic rings from either normotensive or hypertensive rat models. The aim of this present study is to provide an explanation for the observed hemodynamic effects of glycine by employing this *in vitro* study. In these experiments, increasing

concentrations of glycine (100 µM to 3.0 mM) will be used to investigate whether glycine affects basal vascular tone and whether this would be exaggerated in aortic rings isolated from age-matched SHR and L-NAME treated WKY rats. It would be interesting to examine whether the changes in basal tone evoked by glycine are attenuated in the presence of NMDA antagonist(s). Similarly, phenylephrine (PE) constricted rings whether addition of glycine evokes vasodilatation via promoting NO release following activation of endothelial NMDA receptors could be assessed. Such a mechanism could be more active in normotensive rats thereby contributing to the fall in MAP subsequent to glycine administration. In contrast in hypertensive rat models, with the presence of endothelial dysfunction and reduced NO bioavailability, there is exaggerated vascular tone following glycine administration. To summarize, intracisternal administration of glycine promotes a fall in BP that was demonstrated as early as 1972 (Sgaragli et al., 1972; Pavan et al., 1972). Glycine serves a role as a central neurotransmitter. While dietary glycine or chronic administration of glycine could affect hemodynamic responses via alterations in centrally or other peripherally mediated mechanisms, the direct peripheral vascular effects of glycine have not been demonstrated. Thus, it is first of all important to establish whether acute intravenous administration of glycine evokes changes in BP and associated hemodynamic parameters. Based on the above, the following working hypothesis has been formulated.

2.2. Working hypothesis

Acute intravenous administration of glycine evokes depressor response in normotensive WKY rats and a pressor response in hypertensive rats (Mishra et al.,

2008b). This opposing effect of acute glycine induced alteration in MAP is mediated by both vascular and endothelial NMDA receptors. In normotensive WKY rats, with intact functionally active endothelium, pharmacological doses of acute intravenous glycine administration increases NO generation that decreases MAP due to NO-mediated vasodilatation occurring in select regional vascular beds that are sensitive to glycine. The significant level of vasodilatation in these vascular beds leads to reduction in TPR and a concomitant fall in MAP. In contrast, in hypertensive rat models when NMDA receptor mediated endothelial mediators are blunted such as in SHR and L-NAME-induced hypertensive WKY rats, glycine administration might promote increased vascular tone via activation of finite population of NMDA receptors present in VSMCs. This leads to increases in TPR and elevated MAP. Glycine is non polar in nature and might be eliciting its effect by actvating peripheral vascular NMDA reptors present on the endothelium (in normotensive states) to account for this opposite effects of fall in MAP in normotensive and increase in MAP in hypertensive rat models.

2.3. Experimental strategy

Specific experimental objective 1: *Regional hemodynamic study*

To measure the changes in regional blood flow and regional vascular resistance following acute intravenous administration of glycine in age and sex-matched normotensive (14 week old male WKY) and hypertensive rat models (14 week old male SHR and 14 week old L-NAME-treated WKY) using fluorescent microsphere distribution technique. The responses to glycine will be determined in these models either

in the presence or absence of NMDA antagonist, MK-801. All rats were allowed to fast over night.

Specific experimental objective II: *In vivo study*

To measure the changes in systemic hemodynamic parameters such as TPR, MAP, HR, and CO following acute intravenous administration of glycine in age and sex-matched normotensive (14 week old male WKY) and hypertensive rat models (14 week old male SHR and 14 week old L-NAME-treated WKY). In addition, administration of sarcosine, a glycine transporter inhibitor, should augment the depressor and pressor responses to glycine in normotensive and hypertensive rats respectively. Also the changes in MAP, during acute glycine administration will be tested in the presence and absence of at least two different NMDA antagonists, MK-801 and memantine, in all the three rat models will be determined.

Specific experimental objective III: *In vitro study using rat aortic rings*

To determine the changes in basal tone before and after incubation with increasing concentrations of glycine in rat aortic rings isolated from WKY and SHR strain. Also we will examine whether the responses evoked by glycine in vessels of these strains are affected by incubation with optimal concentrations of NMDA antagonist (MK-801). The changes in basal tone induced by glycine will be determined under the above incubation conditions in both endothelium intact and endothelium denuded vessels. In addition, the effect of increasing concentrations of glycine will be evaluated in PE constricted rings. Finally, the goal of this study is to establish whether or not the

observations made in the *in vitro* model correspond/correlate with the changes seen in the *in vivo* model.

2.3.1. Choice of different vascular tissue and different animal models

Different animal models of hypertension have been developed. Although the existing animal models of hypertension are expected to exhibit similar characteristics encountered in human essential hypertension, unfortunately no single hypertensive rat model clearly addresses all the issues. There are two classes of animal models of hypertension developed; genetic and non-genetic models. Spontaneously hypertensive rat (SHR) represents a genetic rat model of hypertension. Non genetic rat models of hypertension are developed either by specific surgical methods or by induction/ inhibition by chemicals and pharmacological agents. Some of the non genetic rat models of hypertension are as follows: Dhal salt sensitive rat strain, deoxycorticosterone (DOCA) treated rats, and chronic NOS inhibitor treated rats. The latter model develops hypertension more similar to the prototype for the non-genetic rat models of hypertension, compared to rats made hypertensive by renovascular surgery and endocrine manipulation (Pinto et al., 1998; Lerman et al., 2005). Gene knock out mouse models have been developed to study different aspects that contribute to metabolic disorders (Yang et al., 1999; Tsutsui et al., 2006). In our study we used two established rat models of hypertension, namely the genetic model of SHR and the other one is chemically induced hypertensive model by chronic treatment with the NOS inhibitor, L-NAME. Both these models share important characteristics that are similar to human essential hypertension (Lerman et al., 2005).

2.3.1.1. Spontaneously hypertensive rat (SHR)

For experimental hypertension research, SHR model serves as an ideal and important animal model since it shares some of the structural modification seen in the vasculature with increased sympathetic activity that is seen in human essential hypertension. They are developed from the outbred normotensive (WKY) rats that were bred with selective hypertensive female WKY rats of the same group (Okamoto et al., 1963). The WKY rat serves as a legitimate control wild type for SHR since the SHR strain was developed from cross mating with siblings with a wide selection from outbred WKY rat. As SHR age, they develop impairment in cardiac function. It is associated with increased coronary vascular resistance and reduced coronary flow reserve and impairment in renal functions that includes glomerular sclerosis, protenuria, glomerular fibrosis and glomerular hypertension (Zhou et al., 2007). There are studies demonstrating increased stiffness and decreased elasticity of the arterial wall in SHR which is also encountered in human essential hypertension (Marque et al., 1999; Bussy et al., 2000). Increased level of oxidative stress with reduced NO availability is another feature seen in the SHR model which is also a common feature in human essential hypertension (Zhou et al., 2008). Due to the impairment in structural and functional integrity of kidney as well as heart with high sympathetic out flow, this contributes to end organ damage associated with high BP in SHR. Alteration in physiological function with structural modification of the vasculature may contribute to progressive increases in vascular resistance and elevated BP which we normally see in human essential hypertension. Due to these similarities, SHR model is widely considered as a suitable experimental animal model for hypertension.

2.3.1.2. Chronic NOS inhibited hypertensive rat

Chronic NOS inhibition by treatment with NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), in drinking water for five days elevates MAP (Ribeiro et al., 1992; Shinde et al., 2005, Desai et al., 2006). In the absence of NO, there is an overactivation of vasoactive contractile factors which are collectively responsible for the increase in vascular resistance and subsequent elevation in MAP. Chronic L-NAME treatment upregulates EDHF activity in rat models (Desai et al., 2006). The pathophysiological alterations that contribute to the impaired function of the heart with wide spread vascular resistance seen in human essential hypertension are exhibited by NOS inhibited hypertensive rats (Lahera et al., 1992). Lahera et al also reported that volume dependent hypertension probably occurs prior to renal impairment in NOS inhibited rat which is one of the cardinal features of human primary hyperetension (Lahera et al., 1992; Guyton et al, 1987). This NOS inhibited hypertensive rat model is a useful tool to evaluate the effects of amino acids in hypertensive rats. Again, it is very convenient, cheaper and less time consuming to develop NOS inhibited hypertensive rats than SHR.

CHAPTER 3. METHODS

3.1. Animals

The present study was approved by our University Review Committee and the protocol conformed to the Guide for the Care and Use of Laboratory Animals stipulated by the Canadian Council on Animal Care and the National Institute of Health (NIH) publication No. 85-23. Two different strains of normotensive rats are used in the present study for comparison. They are 14 week old male Sprague-Dawley (SD) rats (300-350g) and WKY rats (280-320g). Two different models of hypertensive rats are used for the experimental protocol; 14 week old male WKY rats rendered hypertensive by pretreatment with N^G nitro L-arginine methyl ester (L-NAME, 0.7 mg/mL in drinking water ad libitum) for 5 days and SHR (250-270g). All rats were obtained from Charles River (St. Constant, Quebec, Canada).

3.2. Materials

Materials used in the present study are listed as follows: Fluorescent microspheres, glycine, Tween 20, Tween 80, potassium hydroxide (KOH), heparinized saline, saline, cellosolve, phenylephrine hydrochloride (PE), acetylcholine (ACh), N^G nitro L-arginine methyl ester (L-NAME), (+)-5methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), memantine and sarcosine. Microspheres were obtained from Invitrogen, Inc, (Eugene, Oregon, U.S.A.). All other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON., Canada).

Thiopental sodium was obtained from Abbott Laboratories Ltd (Saint Laurent, Quebec, Canada).

3.3. Experimental procedures

The regional hemodynamic studies as well as *in vivo* experiments were conducted in anesthetized rats. All rats were allowed to fast over night. *In vitro* studies were carried out using aortic rings isolated from the same rat strains.

3.3.1. Regional hemodynamic study

3.3.1.1. Surgical procedure for regional hemodynamic study

Rats were anesthetized with *i.v.* thiopental sodium (100 mg/kg, body weight) and body temperature was maintained 37 ^{0}C by putting the rat on an electrical heating pad throughout the surgery period. All the rats were allowed to breathe spontaneously with the insertion of a tracheal tube. The right femoral artery of each rat was cannulated with PE-50 tubing which was then connected to a pressure transducer to measure the MAP and HR using a Power Lab data acquisition system (AD Instruments Inc., Sydney, Australia). The left femoral artery was cannulated with PE-50 tubing and connected to a reciprocal syringe pump (Harvard Apparatus, Quebec, Canada) for collecting reference blood sample during microsphere injection. The left femoral vein was also cannulated with poly ethylene tubing (PE 50) and was used for bolus injection (0.4 ml/kg) of either vehicle (saline) or glycine (prepared in saline and pH was maintained to 7.3 for all the experiments). The final surgical procedure involved the cannulation of right carotid with

PE-50 through the common carotid to the left ventricle. The proper position of the cannula was confirmed with a typical ventricular wave-form (Glenny et al., 1993; Gervais et al., 1999).

3.3.1.2. Measurement of hemodynamic changes using fluorescent microspheres

Blood flow to different organs was measured by using a perfused fluorescent microsphere distribution technique. Four different colors (green, red, red-orange and carmine) of 15 μm diameter fluorescent microspheres (Fluo Spheres®) were purchased and stored at 4 ^{0}C. The colors of microspheres used in a single study were paired on the basis of their absorption/emission maxima wavelength (nm) in order to avoid spill over. Before administration, the microspheres were sonnicated and vortexed heavily to avoid sedimentation. Then it was diluted to a final volume of 0.3 ml with the addition of 0.9 % saline containing 0.01 % w/v Tween 20. This diluted microsphere solution was again vortexed and immediately injected into the left ventricle through the carotid cannula over a period of 20 seconds at the same time when saline/glycine was injected through the left femoral vein. Then the carotid cannula was flushed with saline over a period of 80 seconds to make sure no microsphere was left in the carotid artery. Withdrawal pump (0.5 ml/min) was started 10 seconds before the injection of microspheres/saline to collect reference blood samples for 80 seconds from the femoral artery. Immediately the reference blood sample was transferred to a 10 ml glass tube containing an anticoagulant heparin and placed on ice. The same procedure was followed to collect reference blood samples when glycine (1.0 mmol/kg) was injected through the femoral vein. Different microspheres were used for saline, glycine and MK-801. Then the animal was euthanized

with a high dose of thiopental sodium. Heart, kidneys, spleen, lungs, pancreas and brain were collected as whole and tissues from liver, small intestine, large intestine, skeletal muscle and diaphragm were also collected and placed in cold saline. All tissues were patted dry, transferred to 10 ml glass tubes and weighed. The reference blood samples and tissues were digested in 3-4 ml of ethanolic potassium hydroxide (4.0 M) solution per gram of tissue for 24-48 hours. The tubes were shaken thoroughly every few hours. After 48 hours, the microspheres were recovered by centrifuging at 2000 x g (Precision, Durafuge 100, Winchester, VA) for 20 minutes. Supernatants were carefully removed leaving < 1 ml. The pellet with microspheres was rinsed with 9 ml of 0.25 % Tween 80 in demineralized water at 60 ^{0}C, vortexed, centrifuged again at 2000 x g for 20 min. A final rinse with demineralized water without Tween 80 was carried out and the supernatant was carefully removed leaving <1 ml. To extract dye, 3 ml of cellosolve acetate (2-ethoxy-ethyl acetate) was added to each tube, vortexed thoroughly, wrapped with aluminum foil and allowed to stand for 4 hours to dissolve the microspheres, which were then vortexed and centrifuged again at 2,000 x g for 15 minutes. The supernatant was carefully removed and used for measurement of fluorescence intensity.

3.3.1.3. Measurement of fluorescence

F-2500 fluorescence spectrometer (Hitachi, Tokyo, Japan) was used to measure fluorescence intensity at excitation and emission wavelengths between 350-750 nm using 5 nm slit width. Each individual sample was read in a 5 ml quartz cuvette in duplicate. To determine the blood flow rate per tissue sample (ml/min/g), the following formula was used: Fi = (Ii)(R)/Iref where: Fi = flow to individual sample (ml/min), Ii = fluorescence

intensity of the sample, R = reference sample withdrawal rate (ml/min), Iref = fluorescence intensity of the reference blood sample. MAP (mmHg), HR (beats/min), CO=Total no. of injected microspheres x reference rate (0.5 ml/min) ÷ no. of microspheres in the reference blood sample, TPR (mmHg/ml/min) were calculated (Sampaio et al., 2003, Giancarla et al., 2007).

3.2.2. *In vivo* studies

For *in vivo* study, we used SHR and age matched normotensive Sprague-Dawley (SD), WKY, and WKY rats rendered hypertensive by treatment with NOS inhibitor L-NAME (0.7 mg/mL in drinking water ad libitum) for 5 days that serves as an additional hypertensive model. Rats were anesthetized with *i.v.* thiopental sodium (100 mg/kg, body weight) and body temperature was maintained 37 ^{0}C by putting the rat on an electrical heating pad throughout the experiment. All the rats were allowed to breathe spontaneously through a tracheal cannula. The right femoral artery of each rat was cannulated with PE 50 tubing which was then connected to a pressure transducer to measure the MAP and HR using a Power Lab data acquisition system (AD Instruments Inc., Sydney, Australia). Either left or right femoral vein was cannulated for vehicle and drug administration during the experiment. Volume of vehicle and every dose of drug administered were limited to a volume of 0.4 mL/kg body weight. The pH of all the drugs that were administered was adjusted to 7.3 in all the experiments. A few minutes after cannulation, once the MAP and HR became stabilized, *i.v.* administration of different doses of drug were started. Sufficient time was given between every dose to make sure that the baseline comes back to the resting level. Once the MAP came back to the resting

baseline, the second dose was administered. Saline was used as the vehicle for these experiments. Glycine, MK-801, memantine and sarcosine were dissolved in saline. Bolus *i.v.* administration of vehicle was followed by administration of glycine (1.0 mmol/kg) in all the four rat models. Then, NMDA antagonists, either MK-801 (75 mg/kg), or memantine (50 mg/kg) was slowly infused through *i.p.* injection over a period of 10 to15 min in these rat models (Deng et al., 2002; Rammes et al., 2008). Subsequently 30 min after infusion of MK-801 and 15 min after the infusion of memantine, the responses to glycine was determined. Similarly glycine (1.0 mmol/kg) mediated responses were determined before and 10 min after the slow infusion of the glycine transporter inhibitor, sarcosine (100 mg/kg, *i.p.*) given over a period of 10 min to all groups of rats.

3.3.3. *In vitro* studies

Male WKY rats (300-350 gm) and SHR (250-270 gm) were anesthetized with thiopental sodium (100 mg/kg, *i.v.*) and the thoracic aorta was isolated and placed in cold Kreb's Henseleit buffer. The composition of Kreb's Henseleit buffer (mM) was: 118 NaCl, 4.7 KCl, 1.2 $MgCl_2.5H_2O$, 1.8 $CaCl_2.2H_2O$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 5.5 Glucose. The aorta was cleaned for surrounding fat and mounted in organ bath chamber containing Kreb's solution maintained at 37 ^{0}C with a constant supply of carbogen, which contains 95% oxygen and 5% carbon dioxide. Resting tension (2 gm) was fixed for initial equilibration period of 1 h in Kreb's buffer. After a 60 min wash period, the aortic rings were normalized using standard procedures (bridge amplifier zero). Subsequently the aortic rings were contracted with PE (1 µmol/L). Relaxation of PE constricted vessels to ACh (10 µmol/L) was used to determine endothelial integrity. The endothelium was

considered as denuded if there was no vasodilator response to the addition of a fixed concentration of ACh (10 μmol/L) in the PE constricted vessels. Then, relaxation of PE constricted vessels to glycine (0.1 to 3.0 mmol/L) was examined. The vasodilator responses of glycine (0.1 to 3.0 mmol/L) were determined both in endothelium intact and endothelium denuded vessels of normotensive WKY rats. After a period of 60 min wash, aortic rings were incubated with nitric oxide synthase inhibitor, L-NAME (100 μmol/L) and NMDA receptor antagonist MK-801 (10 μmol/L) separately and the responses to glycine were determined in endothelium intact vessels in WKY rats. Similar procedures were followed to determine the effect of glycine on aortic rings isolated from SHR.

3.4. Statistical analysis

The vasodilator responses were calculated as a percent of each tissue's response to a fixed concentration of PE. Concentration response (CR) curves were computer fitted to nonlinear curve fit (Graph Pad Prism, Graph Pad Software Inc). Maximum relaxation attained after each dose was used to plot the dose-response curve to glycine. Relaxations are expressed as percentage reversal of PE induced tone. All data are shown as mean ± SEM. Then (n = 6) values quoted similarly indicate the number of animals used. Differences of means, between two groups were calculated by student's t-test. In all cases differences were considered significant when $P < 0.05$.

The change in MAP, HR, CO and TPR following glycine administration were plotted as bar diagrams and the data are expressed as mean ± SEM (n = 6). The changes in MAP during glycine administration both in presence and absence of NMDA antagonists were

also plotted and the significance levels of the differences between means were calculated. Changes in MAP during glycine administration before and after the infusion of sarcosine were also determined. Differences of means, between more than two groups were calculated by one way analysis of variance followed by Tukey's post hoc test. The differences were considered significant when $P < 0.05$.

Changes in organ blood flow and organ vascular resistance during glycine administration were calculated and the data are expressed as mean ± SEM (n = 6). The differences between means were calculated by one way analysis of variance followed by Tukey's post hoc test. The differences between means were considered significant when $P < 0.05$.

CHAPTER 4. RESULTS

4.1. Regional hemodynamic study

4.1.1. Comparison of basal systemic hemodynamic parameters between normotensive Sprague-Dawley rats vs. WKY rats

Data pooled from several experiments show that there are no significant differences in systemic hemodynamic parameters such as MAP (112 ± 2 mmHg, 116 ± 5 mmHg), HR (366 ± 5 bpm, 364 ± 9 bpm), CO (108 ± 5 ml/min/gm, 106 ± 5 ml/min/gm), and TPR (1.05 ± 0.2 mmHg/ml/min, 1.09 ± 0.08 mmHg/ml/min) values determined in two groups of age-matched male Sprague-Dawley rats and WKY rats (n = 6 per each group) respectively (Table 3).

4.1.2. Normotensive WKY rats vs. hypertensive rat models

The basal MAP values were significantly higher ($P < 0.01$) in both L-NAME treated WKY rats (158 ± 3 mmHg) and SHR (183 ± 7 mmHg) compared to values determined in WKY (116 ± 5 mmHg) group (Figure 2, and Table 3). A significant decrease ($P < 0.01$) in CO (77 ± 3 ml/min/gm) was noted in L-NAME treated WKY compared to CO values in normotensive WKY rats (106 ± 5 ml/min/gm) (Figure 3, and Table 3). This pattern of increase ($P < 0.01$) in MAP and decrease ($P < 0.05$) in CO was also significant in the genetic model of SHR strain, (83 ± 2 ml/min/gm) compared to CO values noted in normotensive WKY (106 ± 5 ml/min/gm) group (Figure 3, and Table 3).

However, the HR values were significantly higher ($P < 0.05$) in SHR (383 ± 6 bpm) group compared to WKY (363 ± 9 bpm) group (Figure 4 and Table 3). The HR values were not significantly different between L-NAME treated WKY (349 ± 8 bpm) and WKY (363 ± 9 bpm) group (Figure 4 and Table 3).

4.1.3. L-NAME treated WKY rats vs. SHR

A significant difference ($P < 0.01$) in HR values was noted between L-NAME treated WKY (349 ± 8 bpm) and SHR (383 ± 6 bpm) group (Figure 4 and Table 3). Moreover, the MAP was much higher ($P < 0.05$) in SHR (183 ± 7mmHg) compared to MAP (158 ± 3 mmHg) values seen in L- NAME treated WKY group (Figure 2 and Table 3).

4.2. Comparison of glycine induced changes in systemic parameters

4.2.1. Normotensive Sprague-Dawley rats vs. WKY rats

Data pooled from several experiments show that there was no significant differences in systemic hemodynamic parameters, such as MAP (79 ± 6 mmHg vs. 76 ± 8 mmHg), HR (378 ± 4 bpm vs. 386 ± 7 bpm), CO (125 ± 6 ml/min/gm vs. 126 ± 5 ml/min/gm) and TPR (0.5 ± 0.07 mmHg/ml/min vs. 0.6 ± 0.04 mmHg/ml/min) between the two normotensive groups, Sprague-Dawley and WKY rats respectively, following acute glycine (1 mmol/kg) administration (Table 3).

4.2.2. Normotensive WKY rats vs. hypertensive rat models

Acute *i.v.* glycine administration led to significant changes in several hemodynamic parameters in both normotensive WKY and L-NAME treated WKY and

SHR strains. MAP (76 ± 8 mmHg) decreased ($P < 0.01$) and CO (126 ± 5 ml/min/g) increased significantly ($P < 0.01$) in WKY rats compared to basal MAP (116 ± 5 mmHg) and CO (106 ± 5 ml/min/g) values noted prior to glycine administration (Figure 5, Figure 6, and Table 3). However, there was no significant difference in HR values before (363 ± 9 bpm) and after (386 ± 9 bpm) glycine administration in WKY rats (Figure 7). However, TPR (0.6 ± 0.04 mmHg/ml/min) decreased significantly ($P < 0.05$) during glycine administration compared to TPR (1.09 ± 0.08 mmHg/ml/min) during vehicle administration (Table 3).

In hypertensive L-NAME treated rats, there was a significant increase ($P < 0.05$) in MAP (182 ± 6 mmHg) and decrease ($P < 0.05$) in CO (64 ± 3 ml/min/gm) following acute glycine administration, compared to MAP (158 ± 3 mmHg) and CO (77 ± 3 ml/min/gm) following vehicle administration while there was no significant change (349 ± 9 bpm vs 334 ± 9 bpm) in HR (Figure 5, Figure 6, Figure 7, and Table 3). TPR (2.8 ± 0.19 mmHg/ml/min) increased significantly ($P < 0.01$) with glycine administration compared to the baseline TPR (2.05 ± 0.16 mmHg/ml/min), following vehicle administration, in L-NAME treated WKY rats (Table 3). The saline administration (vehicle) did not evoke any significant change from baseline parameters for MAP, CO, HR, and TPR while differences were remarkable following glycine administration.

In SHR group, the basal MAP was higher (210 ± 6 mmHg) and CO was lower (63 ± 3 ml/min/gm), compared to the baseline MAP (183 ± 7 mmHg) and CO (83 ± 2 ml/min/gm). The differences in MAP ($P < 0.05$) and CO ($P < 0.05$) were statistically

significant (Figure 5, Figure 6, and Table 3). HR did not show any significant change (383 ± 6 bpm vs. 360 ± 6 bpm) in SHR following glycine administration compared to vehicle administration (Figure 7 and Table 3). The TPR (3.3 ± 0.24 mmHg/ml/min) increased significantly ($P < 0.01$) following acute glycine administration, compared to the resting TPR (2.2 ±0.11 mmHg/ml/min) values noted in the same group of rats (Table 3).

4.2.3. L-NAME treated WKY rats vs. SHR

No significant change was observed in systemic hemodynamic parameters such as MAP (182 ± 6 mmHg vs. 210 ± 6 mmHg), HR (334 ± 9 bpm vs. 360 ± 6 bpm), CO (64 ± 3 ml/min/gm vs. 63 ± 3 ml/min/gm) and TPR (2.8 ± 0.19 mmHg/ml/min vs. 3.3 ± 0.24 mmHg/ml/min) between the two hypertensive rat models, L-NAME treated hypertensive WKY and SHR following acute glycine administration (Table 3).

4.3. Comparison of regional blood flow between normotensive WKY rats vs. hypertensive rat models

We found a significant fall in MAP during glycine (1 mmol/kg) administration compared to vehicle administration in normotensive WKY rats (Figure 8A). This fall in MAP was blocked in the presence of MK-801 (Figure 8 A). However in L-NAME treated WKY group glycine evoked increase in MAP was blocked in the presence of MK-801 (Figure 8 B). Similarly, in hypertensive SHR strain glycine did not increase MAP 45 min after the incubation of MK-801 (Figure 8 C). Data pooled from several experiments

revealed that there were significant changes in the blood flow values between normotensive and hypertensive rats during saline/glycine administration (Table 4).

4.3.1. Comparison of basal blood flow - WKY rats vs. hypertensive rat models

A significant difference in basal blood flow to different organs/tissues in hypertensive rats was noted in comparison to normotensive WKY rats. Acute vehicle administration (saline, 0.4 mL/kg) *per se* did not modify the basal blood flow to various organs/tissues occurred in the following rank order with the highest level of blood flow to kidneys; kidneys> heart> brain> pancreas> diaphragm> small intestine> large intestine> stomach> spleen> skeletal muscle (Table 4). The baseline blood flow to either kidney was similar with a difference of < 5% in all the three rat models. In WKY rats, the kidneys received the highest blood flow, followed by heart and brain (Table 4). In L-NAME treated WKY, the basal blood flow calculated was highest in kidneys> heart> brain> diaphragm> spleen> stomach> small intestine> pancreas> skeletal muscle> large intestine (Table 4). In SHR, the basal blood flow was highest in the following rank order heart> kidney> brain> skeletal muscle> small intestine> pancreas> stomach> large intestine> diaphragm (Table 4). In SHR strain, the basal blood flow to kidneys was much less compared to the basal blood flow to kidneys in WKY rats, and the difference is significant ($P < 0.001$, Table 4).

4.3.2. Comparison of basal blood flow - L-NAME treated WKY rats vs. SHR

In SHR, there was a significant decrease in blood flow to kidneys when compared to the blood flow in L-NAME treated hypertensive WKY rats and the difference in values

between these two strains was significant ($P < 0.001$) (Table 4). Blood flow to spleen was also significantly lower ($P < 0.05$) in SHR compared to the blood flow to spleen in L-NAME treated WKY rats (Table 4).

4.4. Comparison of glycine induced changes in regional blood flow

4.4.1. Normotensive WKY rats vs. hypertensive rat models

A significant change in blood flow to most vital organs were noted following acute glycine (1 mmol/kg) administration among and between normotensive as well as hypertensive rat models. In normotensive WKY rats, there was a significant increase in blood flow to kidneys, heart and brain after glycine administration compared to acute vehicle administration (Figure 9 A and Table 4). Acute administration of glycine increased blood flow to left vs. right kidneys, (188%) and (192%) respectively compared to vehicle in WKY rats. The differences of means were significant ($P < 0.01$, Figure 9 A, Table 4). We observed an increase in blood flow to heart (160%), after glycine administration compared to baseline blood flow to heart and the difference was significant ($P < 0.05$, Figure 9 A, Table 4). Blood flow to brain was also increased (59%), following glycine administration compared to vehicle administration, and this increase is significant ($P < 0.05$, Figure 9 A and Table 4). We did not find any significant change in blood flow to pancreas, diaphragm, skeletal muscle, spleen, stomach, small intestine and large intestine following glycine administration, compared to vehicle administration (Figure 9 A, Figure 9 B and Table 4).

In L-NAME treated WKY rats glycine evoked a significant decrease ($P < 0.01$) in blood flow to kidneys (46%) and (47%), heart (46%) and brain (35%) compared to blood flow following vehicle administration in the same organs (Figure 10 A, Table 4). Blood flow to pancreas (61%) and diaphragm (33%) were also significantly decreased ($P < 0.05$) compared to blood flow following vehicle administration in pancreas and diaphragm (Figure 10 A and Table 4). However, acute glycine administration did not alter the blood flow to the following organs, such as skeletal muscle, spleen, stomach, small intestine and large intestine compared to vehicle administration (Fig. 10 A and Table 4).

In SHR, acute glycine administration significantly decreased ($P < 0.05$) blood flow to kidneys left vs. right, (51%) and (57%) respectively, heart (71%), brain (55%), pancreas (47%) and skeletal muscle (57%) compared to vehicle administration in the same organs (Figure 11 A and Table 4). Blood flow to following organs/tissues such as, diaphragm, spleen, stomach, small intestine and large intestine were not significantly different after glycine administration compared to vehicle administration (Figure 11 B and Table 4). While acute glycine administration decreased the blood flow to several vital organs, its effect on changes in blood flow in splanchnic vascular beds was not significant.

4.4.2. L-NAME treated WKY rats vs. SHR

In L-NAME treated WKY rats, administration of glycine significantly decreased blood flow ($P < 0.05$) to diaphragm compared to vehicle administration whereas, in the SHR group glycine significantly decreased blood flow ($P < 0.05$) to skeletal muscle compared to vehicle (Table 4).

4.5. Changes in regional blood flow before and after glycine administration in presence of MK-801

In normotensive WKY rats glycine induced fall in MAP and in hypertensive L-NAME treated WKY rats and SHR strain glycine induced increase in MAP were blocked in the presence of NMDA antagonist MK-801 (Figure 12 A, B, C). This increase and decrease in MAP were associated with increase and decrease in blood flow to different vascular beds in these rat models. In the normotensive WKY rats acute glycine administration increased blood flow to vital organs such as heart, kidney and brain compared to vehicle administration whereas in presence of MK-801 the same dose of glycine did not produce any significant change in blood flow to the same organs (Figure 13 A). Glycine did not show any significant difference in blood flow to stomach, small intestine, large intestine and spleen in the presence of MK-801 in WKY rats (Figure 13 B). In L-NAME treated WKY glycine decreased the blood flow to heart, kidneys, brain, pancreas and diaphragm (Figure 14 A). This response was blocked in the presence of MK-801 (Figure 14 A). Glycine did not any significant change in blood flow to stomach, small intestine, large intestine and spleen either in the presence or absence of MK-801 in L-NAME treated rats (Figure 14 B). Similarly, in SHR glycine mediated decrease in blood flow to heart, kidneys, brain, pancreas and skeletal muscle were blocked completely in presence of MK-801(Figure 15 A). In the splanchnic vascular bed glycine did not show any significant change in the blood flow both in the presence or absence of MK-801 compared to blood flow during saline administration in SHR strain (Figure 15 B). This result provides an important information that MK-801, a non selective NMDA

receptor antagonist completely blocked glycine mediated increase and decrease in blood flow to several organs/tissues in normotensive WKY rats and hypertensive rat models.

4.6. Comparison of basal organ vascular resistance

Since there was wide variation observed in basal regional blood flow to various organs/tissues in all strains, we calculated the baseline organ vascular resistance values, a measurement that is inversely associated with blood flow. The values calculated varied significantly in normotensive WKY rats, L-NAME treated hypertensive WKY rats and SHR groups (Table 5).

4.6.1. Normotensive WKY rats vs. hypertensive rat models

Data gathered from several experiments revealed that in hypertensive rat models basal peripheral organ vascular resistance was elevated in some organs/tissues compared to normotensive WKY rats. In L-NAME treated hypertensive WKY rats organ vascular resistance increased significantly in different organs/tissues in following rank order ($P < 0.001$) in large intestine, ($P < 0.01$) in pancreas, ($P < 0.05$) in spleen compared to normotensive WKY rats (Table 5). Whereas organ vascular resistance significantly decreased ($P < 0.001$) in diaphragm and ($P < 0.05$) in brain compared to normotensive WKY rats (Table 5). Interestingly, in SHR group, the overall organ vascular resistance was found to be higher than L-NAME treated hypertensive rats and increased significantly to the following organs/tissues ($P < 0.001$) such as, kidneys, ($P < 0.001$) spleen, ($P < 0.01$) pancreas, ($P < 0.01$) small intestine and ($P < 0.05$) heart compared to normotensive WKY group (Table 5).

4.6.2. L-NAME pretreated hypertensive WKY rats vs. SHR

Although we found an increase in organ vascular resistance in both L-NAME pretreated hypertensive WKY and SHR strain, clearly overall vascular resistance was found to be higher in many organs/tissues in SHR compared to L-NAME treated hypertensive WKY rats (Table 5). In SHR, there is an wide spread increase in organ vascular resistance to following organs/tissues ($P < 0.01$) in kidneys, ($P < 0.01$) in spleen, ($P < 0.05$) in diaphragm, compared to L-NAME pretreated hypertensive WKY group (Table 5).

4.7. Glycine Induced changes in organ vascular resistance

4.7.1. Normotensive WKY rats vs. hypertensive rats

Following glycine administration, in normotensive WKY rats organ vascular resistance decreased significantly ($P < 0.001$) in heart, kidneys, brain and diaphragm compared to the vascular resistance seen in the same organs after vehicle administration (Table 5). However, in both the hypertensive rat models, the organ vascular resistance increased significantly following acute glycine administration compared to vehicle administration. In L-NAME treated WKY rats, glycine administration evoked an increase ($P < 0.001$) in the vascular resistance in brain, pancreas, diaphragm, ($P < 0.01$) kidneys, and ($P < 0.05$) heart compared to vehical administration in the respective organs (Table 5). Alternately, in SHR strain, vascular resistance increased significantly ($P < 0.001$) in heart, kidneys, brain, diaphragm, skeletal muscle and ($P < 0.01$) pancreas following

glycine administration compared to vehicle administration in the same group of rats (Table 5).

4.7.2. L-NAME pretreated hypertensive WKY rats vs. SHR

Organ vascular resistance is significantly higher in hypertensive rat models. However, increases in resistance in individual organs/tissues are not similar between L-NAME treated WKY and SHR groups (Table 5). Glycine-evoked increase in vascular resistance in hypertensive rat models targets several different organs, although some of them are common. In L-NAME treated hypertensive rats glycine targets following organs/tissues where the vascular resistance values are significantly higher ($P < 0.001$) in pancreas, ($P < 0.05$) brain, heart and diaphragm compared to SHR strain (Table 5). However, in SHR, vascular resistance values are found to be significantly higher ($P < 0.05$) in kidneys compared to L-NAME treated group (Table 5). In addition, skeletal muscle vascular resistance was increased significantly ($P < 0.001$) after glycine administration in SHR but not in chronic L-NAME treated WKY (Table 5).

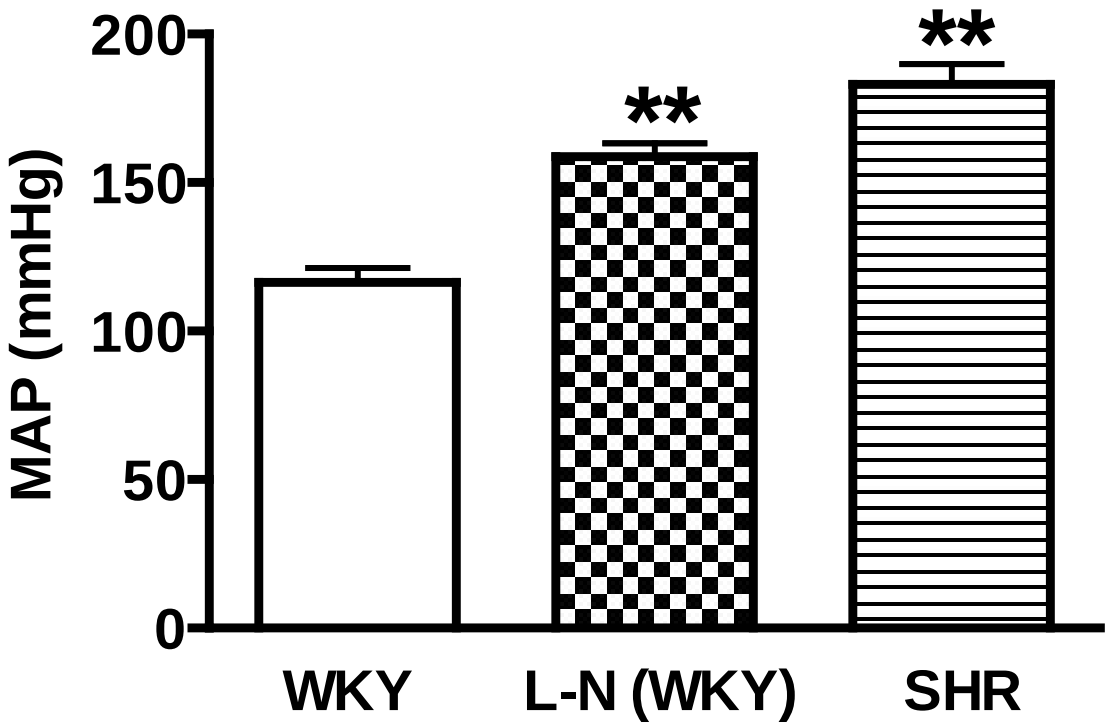

Figure 2. The bar diagram compares the basal MAP (mmHg) levels in 14 week old male WKY, chronic L-NAME (L-N) treated WKY and SHR strains. Each bar represents mean ± SEM values (n = 6 rats/group).

**p < 0.01 compared to WKY group.

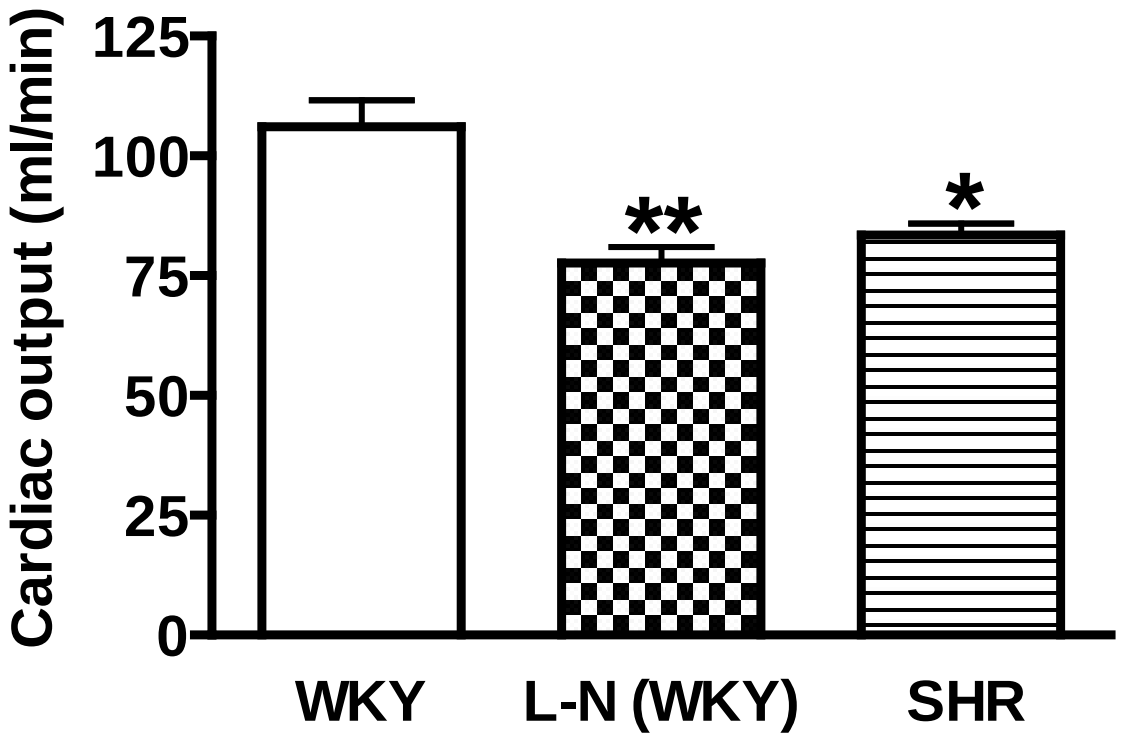

Figure 3. The bar diagram compares the basal cardiac output (ml/min) in14 week old WKY, chronic L-NAME treated WKY (L-N) and SHR strains. Each bar represents mean ± SEM values (n = 6 rats/group).

*$p < 0.05$ and **$p < 0.01$ compared to WKY group.

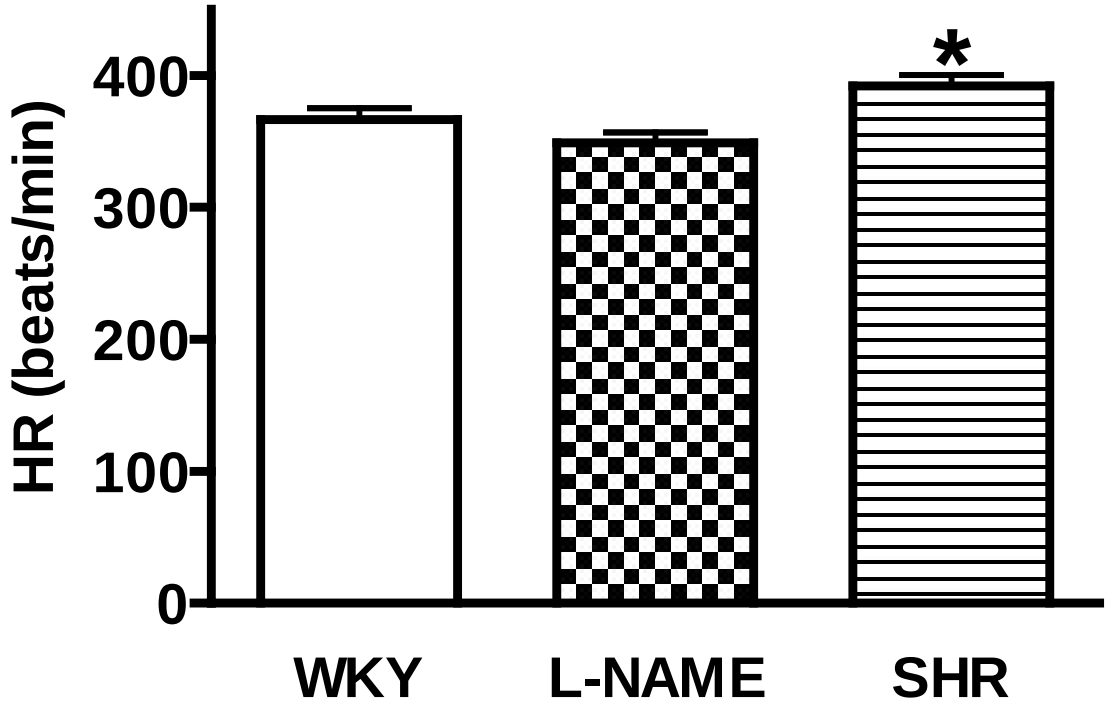

Figure 4. The bar diagram compares the basal heart rate values (beats/min) in 14 week old WKY, chronic L-NAME treated WKY and SHR strains. Each bar represents mean ± SEM values (n = 6 rats/group).

*$p < 0.05$ compared to WKY group.

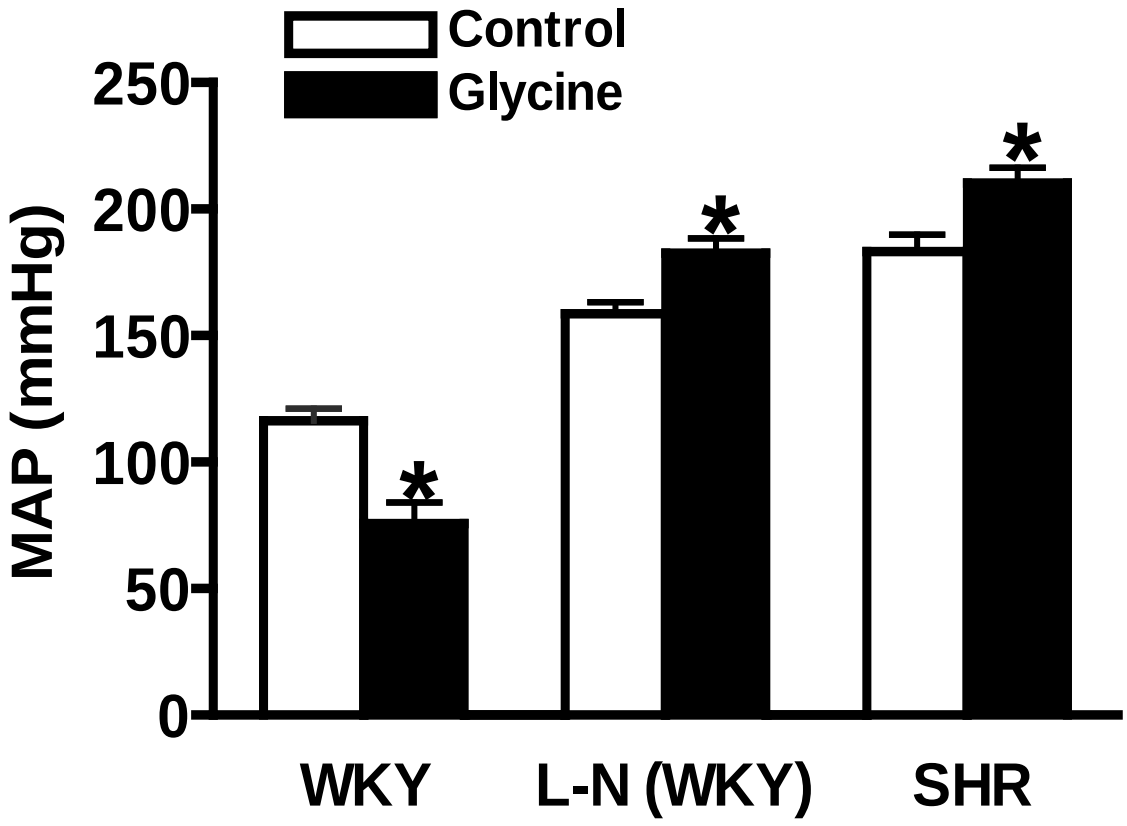

Figure 5. The bar diagram compares the MAP (mmHg) before and after acute glycine administration (1.0 mmol/kg, *i.v.*) in 14 week old WKY, chronic L-NAME treated WKY (L-N) and SHR strains. Each bar represents mean ± SEM values (n = 6 rats/group).

*$p < 0.05$ compared to respective control group.

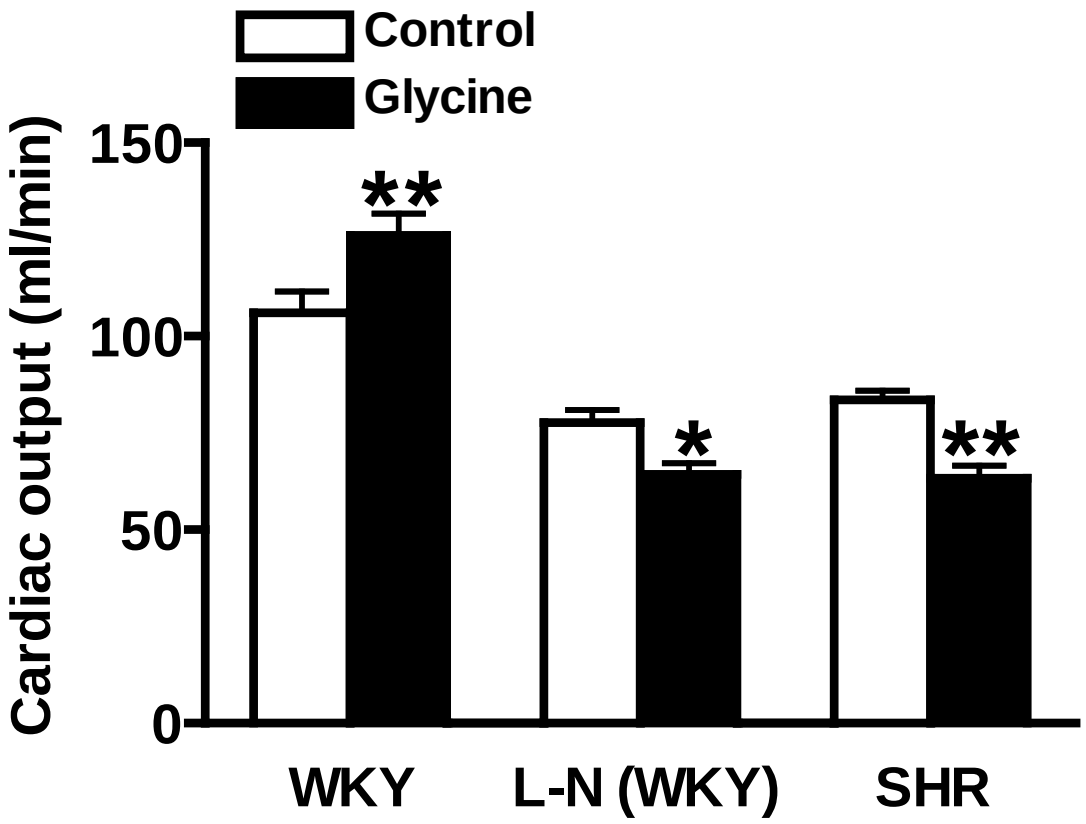

Figure 6. The bar diagram compares the cardiac output before and after acute glycine administration (1.0 mmol/kg, *i.v.*) 14 week old WKY, chronic L-NAME (L-N) treated WKY and SHR strains. Each bar represents mean ± SEM values (n = 6 rats/group).

*$p < 0.05$, **$p < 0.01$ compared to respective control group.

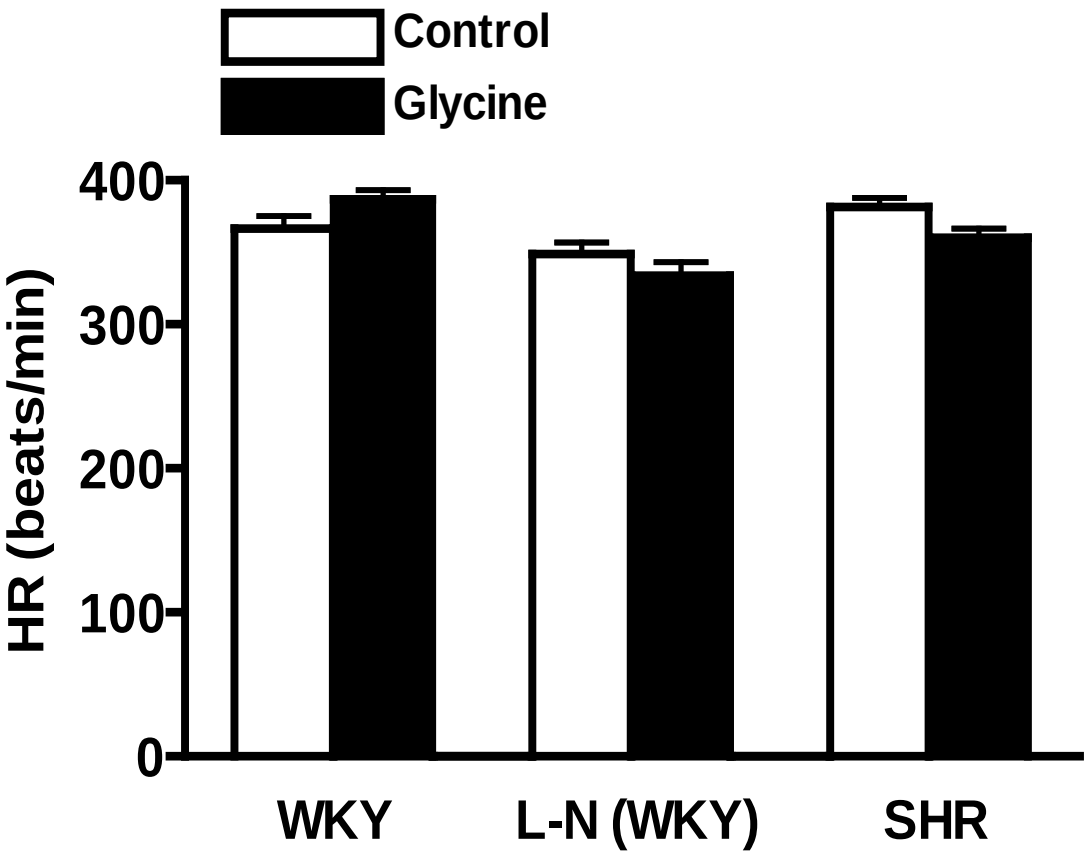

Figure 7. The bar diagram compares the HR before and after acute glycine administration (1.0 mmol/kg, *i.v.*) in 14 week old WKY, chronic L-NAME (L-N) treated WKY and SHR strains. Each bar represents mean ± SEM values (n = 6 rats/group).

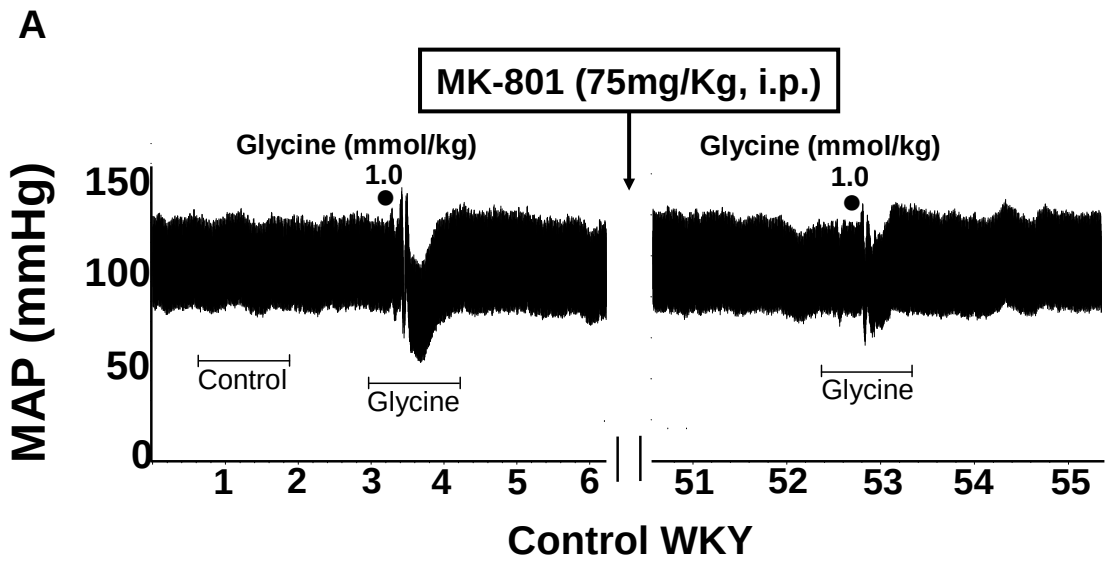

Figure 8 A. A typical representative experiment that shows the fall in MAP to acute administration of glycine before and after slow infusion of MK-801 (75 mg/kg, *i.p.*). MK-801 blocks glycine evoked response 45 minute after its intraperitonial administration in normotensive WKY rats (n = 6 rats).

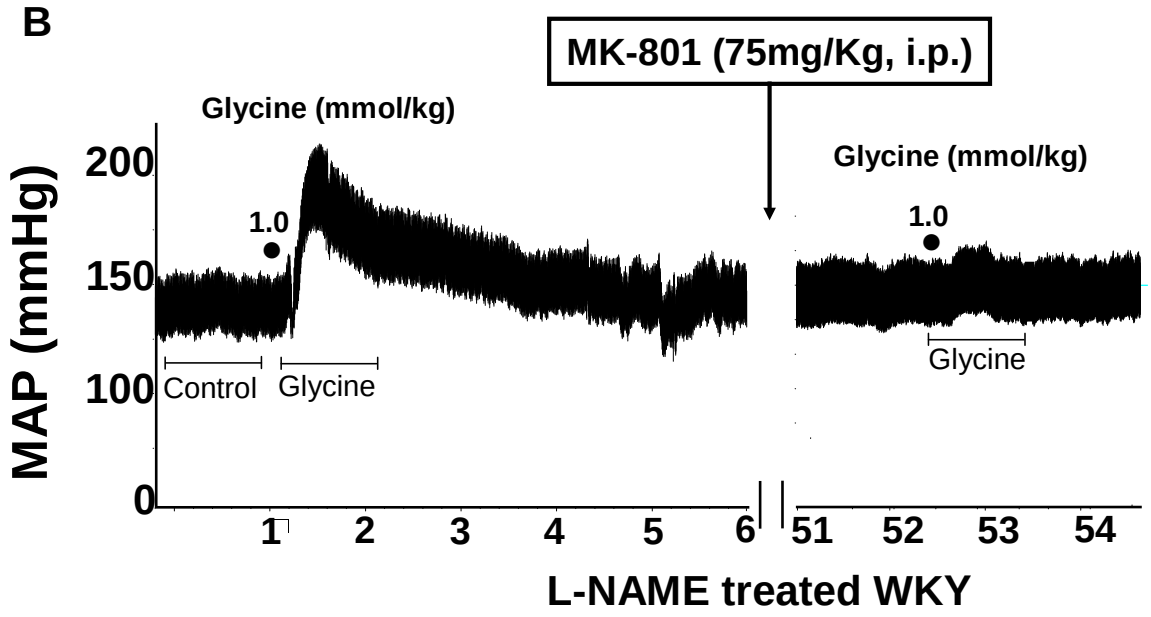

Figure 8 B. A typical representative experiment that shows acute glycine (1 mmol/kg) administration elevates MAP and MK-801 (75 mg/kg, *i.p.*) blocks the response in chronic L-NAME treated WKY rats (n = 6 rats).

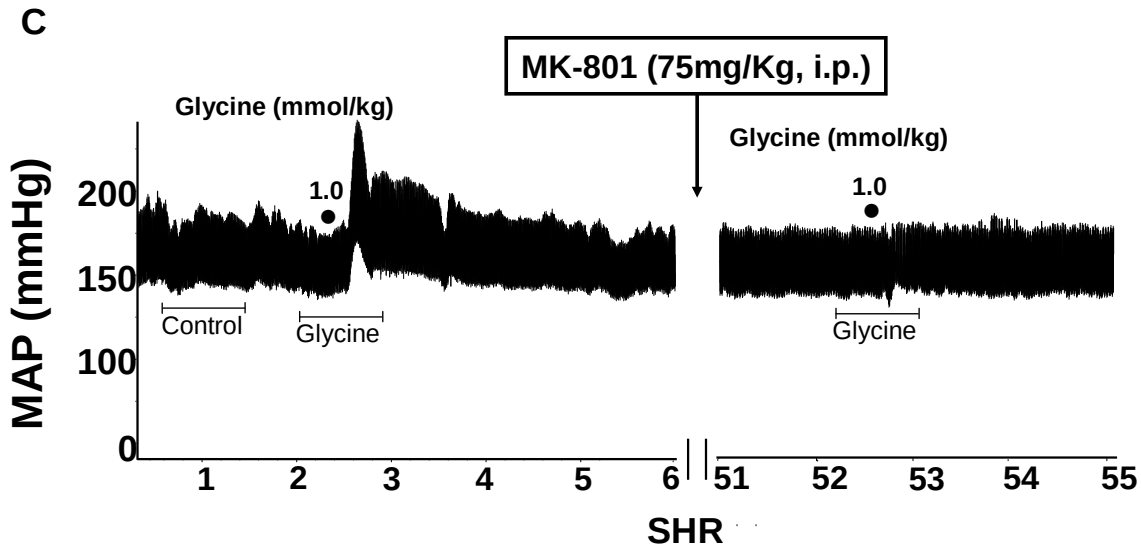

Figure 8 C. A typical representative experiment that compares the increase in MAP following acute glycine administration (1.0 mmol/kg, *i.v.*) and lack of pressor response to same dose of glycine after administration of MK-801 (75 mg/kg, *i.p.*) in SHR strains (n = 7 rats).

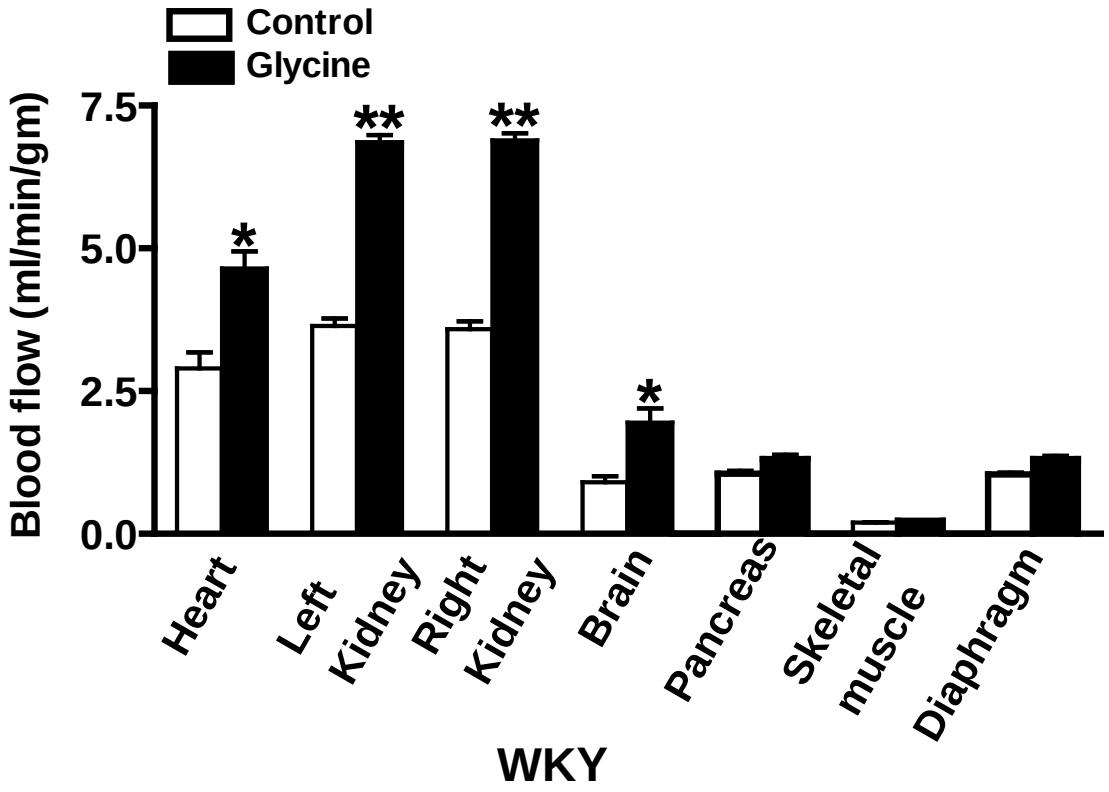

Figure 9 A. Comparison of blood flow to different organs/tissues (heart, left kidney, right kidney, brain, pancreas, skeletal muscle and diaphragm) following acute glycine (1.0 mmol/kg, *i.v*) administration in 14 week old WKY rats. Each bar represents mean ± SEM values (n = 6 rats).

*$p < 0.05$, **$p < 0.01$ compared to respective control value.

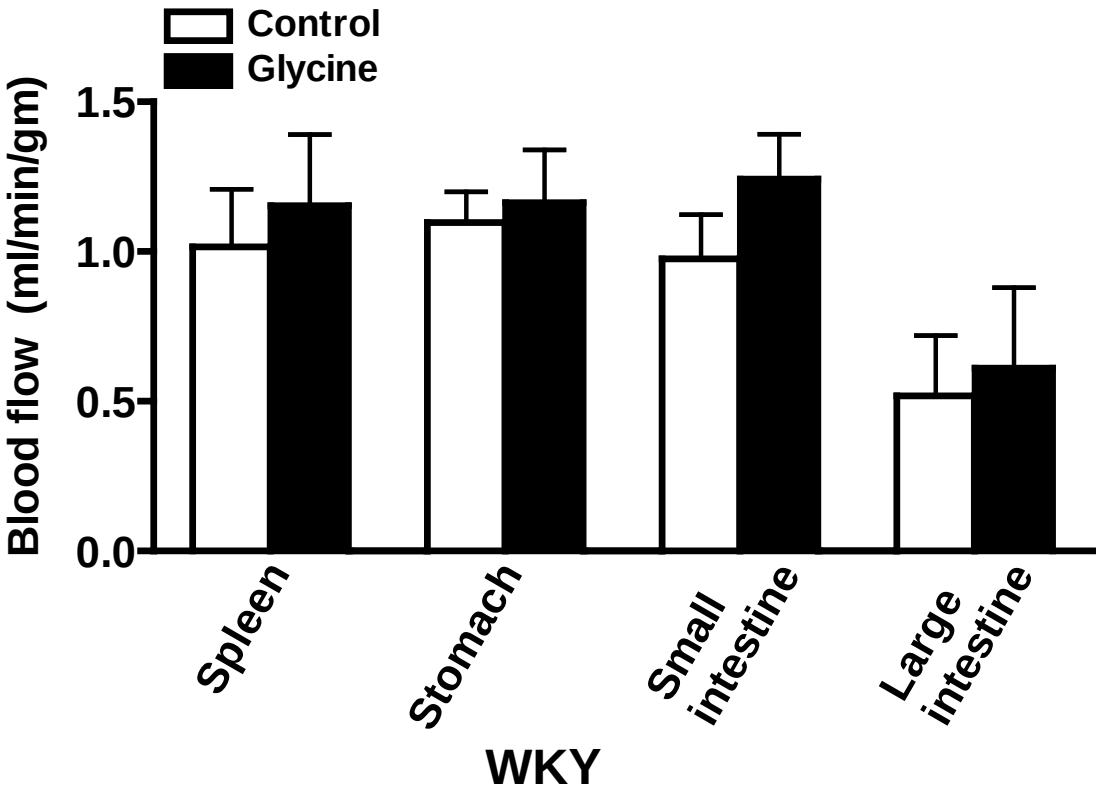

Figure 9 B. Comparison of regional blood flow (ml/min/gm) to different organs/tissues (spleen, stomach, small intestine and large intestine) following acute glycine (1.0 mmol/kg, *i.v*) administration in 14 week old WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

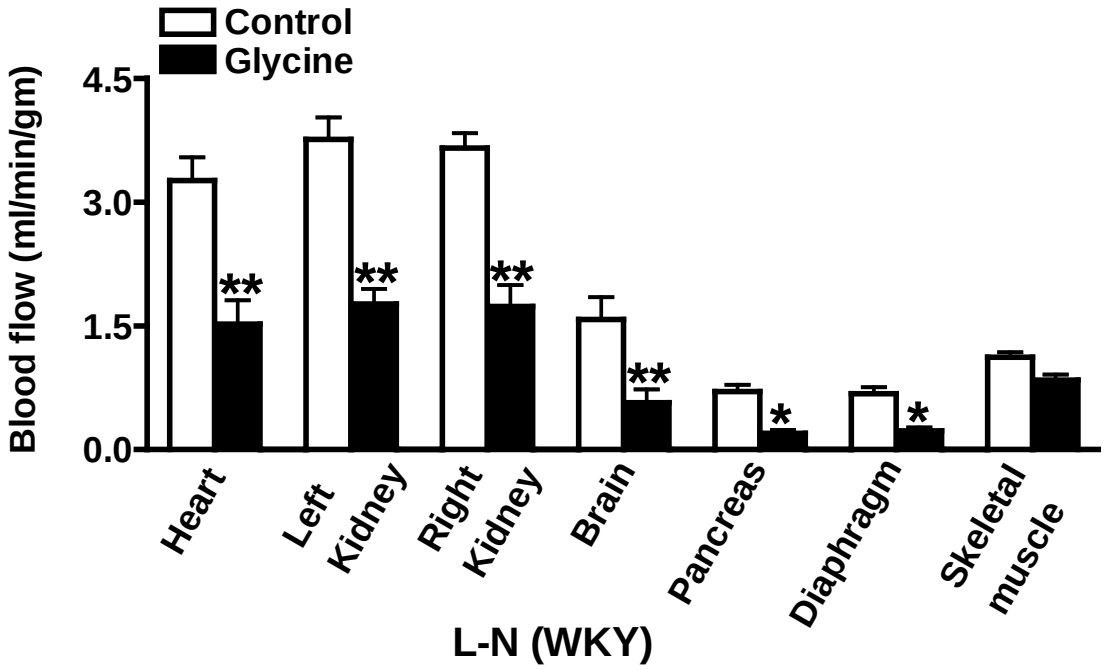

Figure 10 A. Comparison of regional blood flow (ml/min/gm) to different organs/tissues (heart, left kidney, right kidney, brain, pancreas, diaphragm and skeletal muscle) following acute glycine administration (1.0 mmol/kg, *i.v*) in 14 week old chronic L-NAME (L-N) treated WKY rats. Each bar represents mean ± SEM value (n = 6 rats). *$p < 0.05$, **$p < 0.01$ compared to respective control.

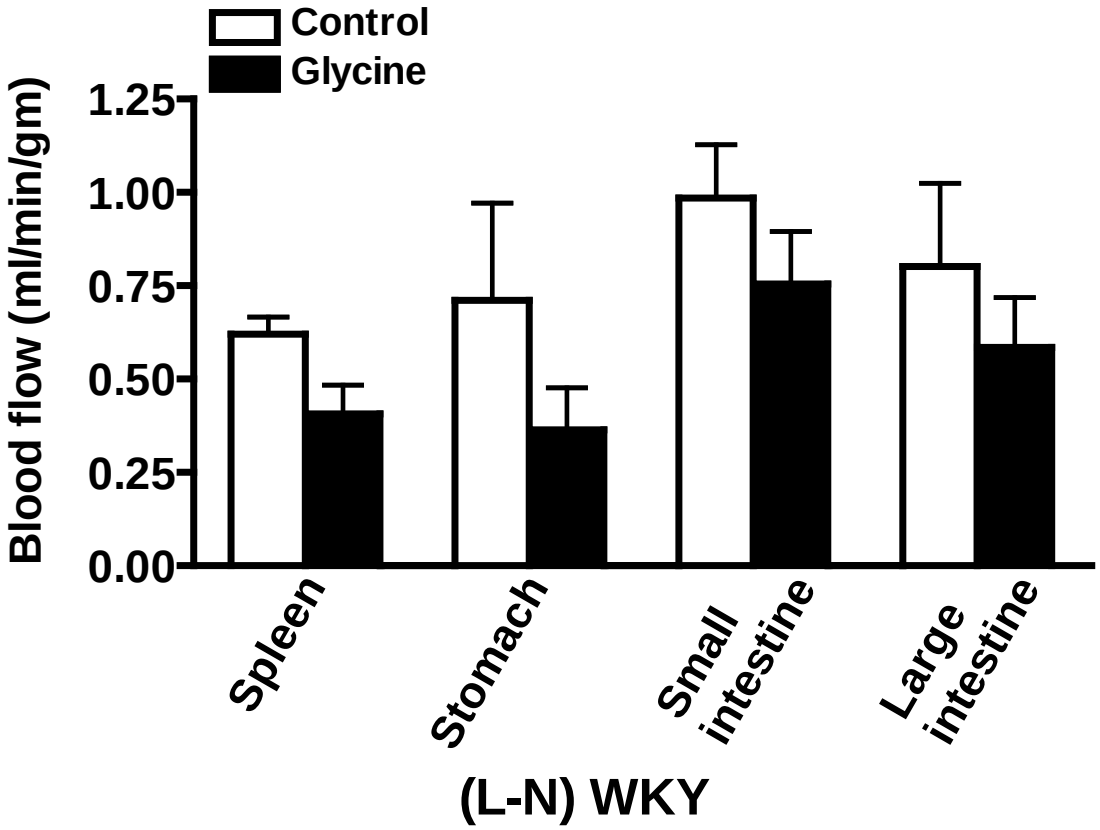

Figure 10 B. Comparison of regional blood flow to different organs/tissues (spleen, stomach, small intestine and large intestine) after acute glycine administration (1.0 mmol/kg, *i.v.*) in 14 week old chronic L-NAME treated WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

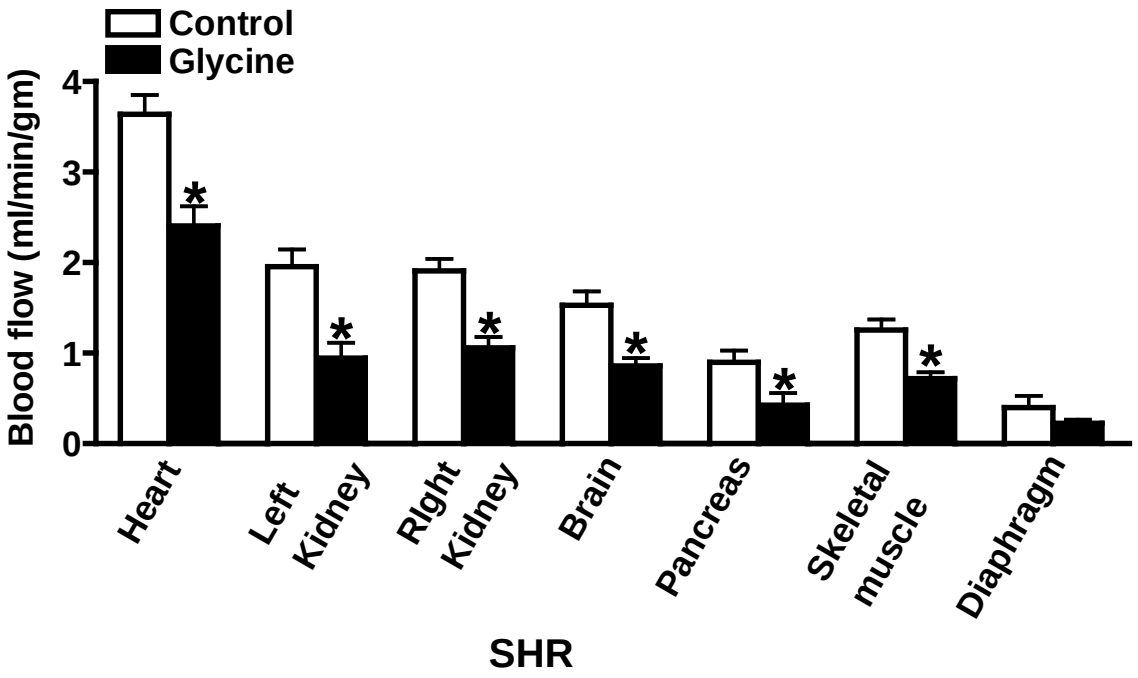

Figure 11 A. Comparison of the regional blood flow (ml/min/gm) to different organs/tissues (heart, left kidney, right kidney, brain, pancreas, skeletal muscle and diaphragm) following acute glycine infusion (1.0 mmol/kg, *i.v.)* in 14 week old SHR. Each bar represents mean ± SEM value (n = 6 rats).

*$p < 0.05$ compared to respective control.

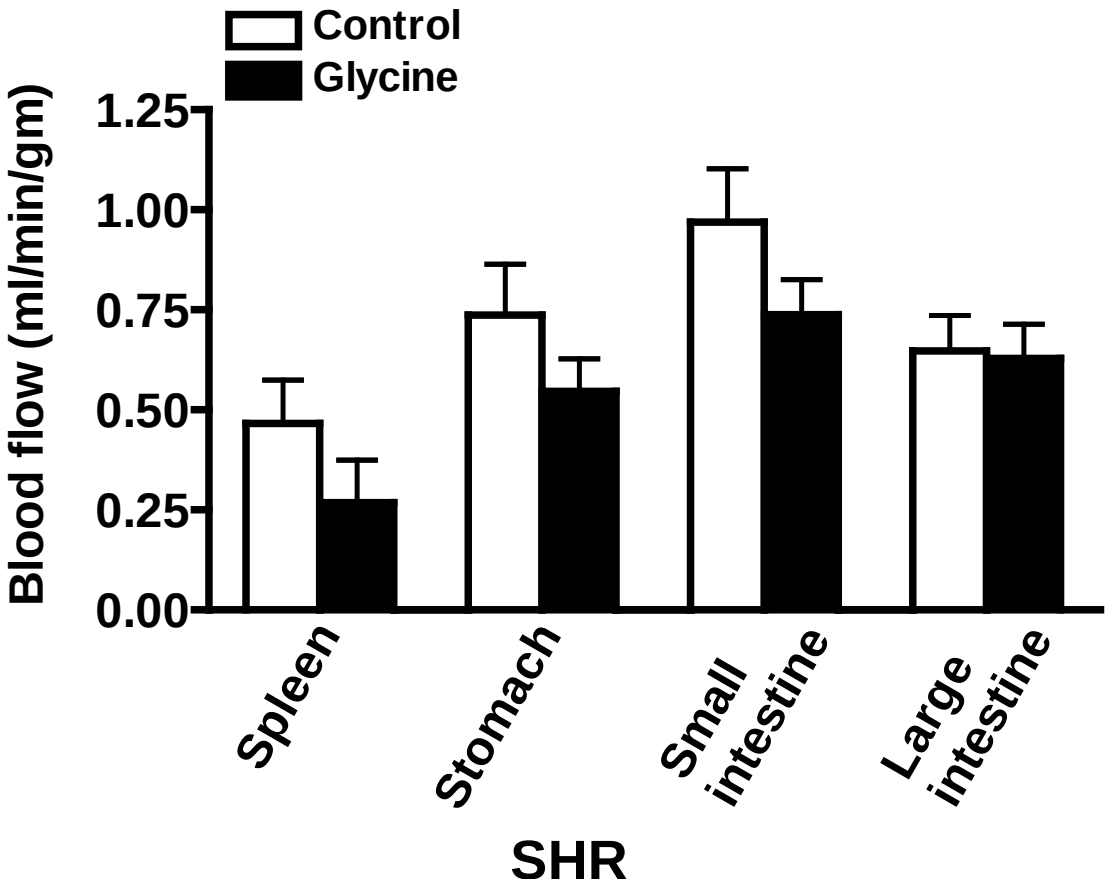

Figure 11 B. Comparison of the regional blood flow (ml/min/gm) to following tissues (spleen, stomach, small intestine and large intestine) after acute glycine administration (1.0 mmol/kg, *i.v.*) in 14 week old SHR. Each bar represents mean ± SEM value (n = 6 rats).

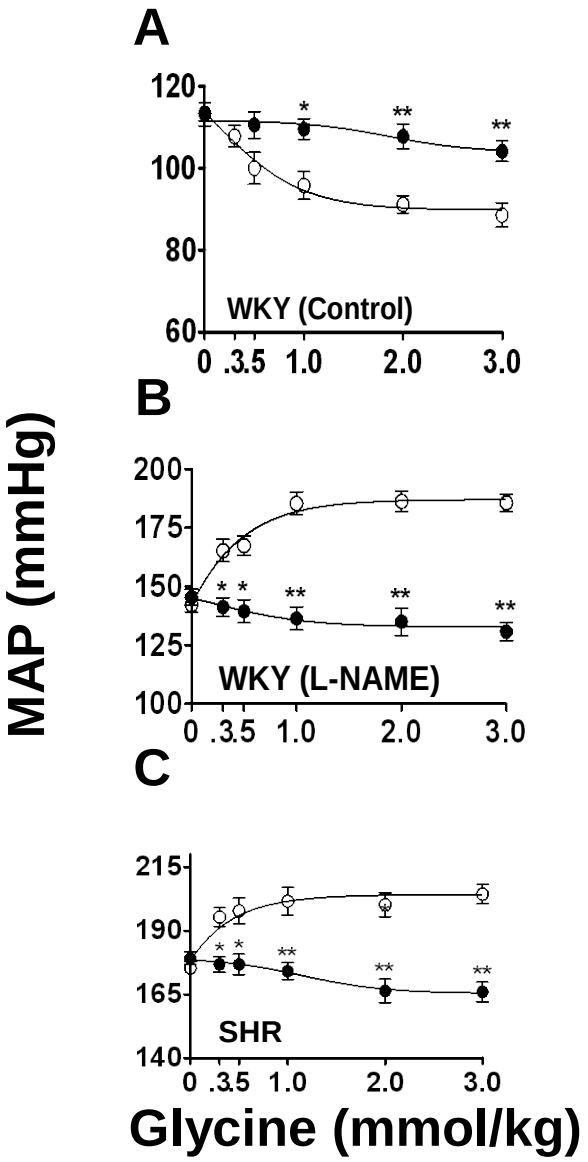

Figure 12. The dose-response curves compare MAP (mmHg) evoked by increasing concentration of glycine (0.3 to 3.0 mmol/kg), either before (○) and after (●) slow infusion of MK-801(75 mg/kg, i.p) administration in WKY (A), chronic L-NAME treated WKY (B) and SHR (C) strains. Each data point is mean ± SEM values (*n* = 6 rats/group).

*p < 0.05, **p < 0.01 compared to respective control in each group.

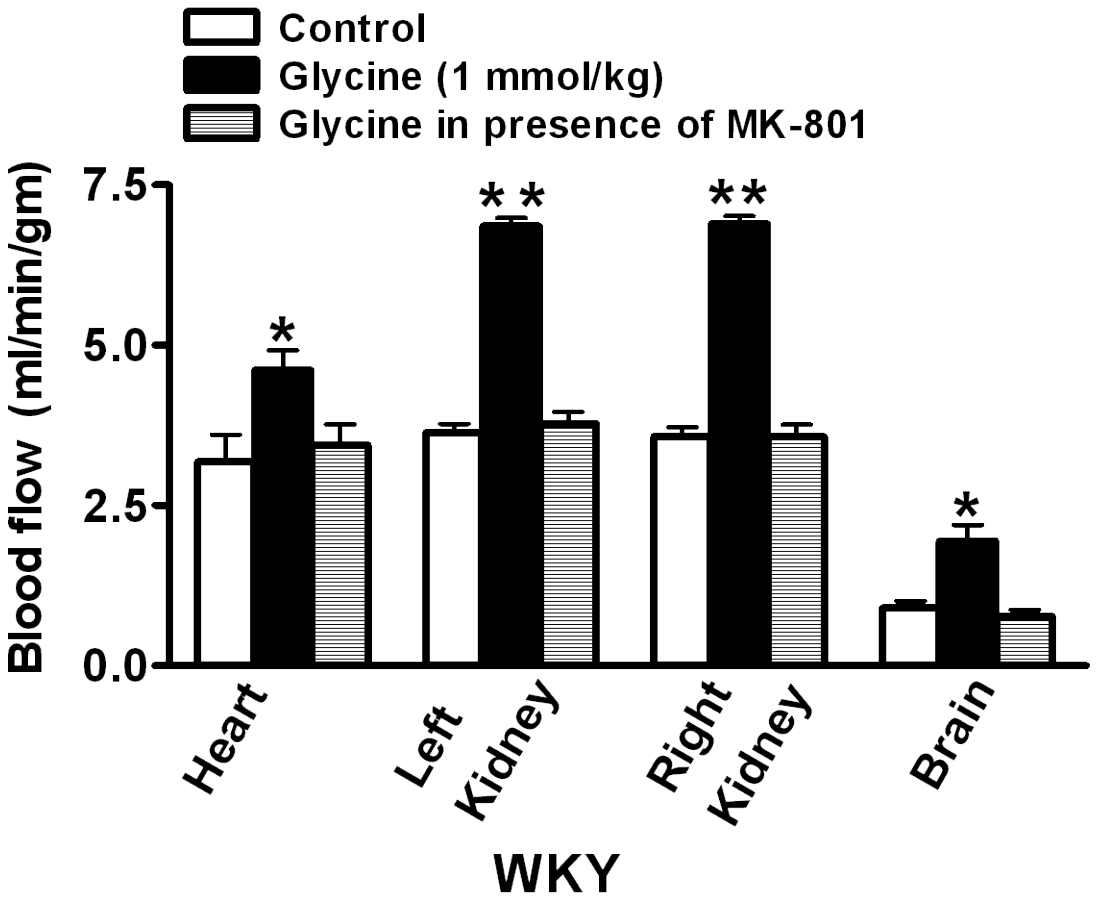

Figure 13 A. Bar diagram compares the blood flow (ml/min/gm) attained in various tissues/organs (heart, left kidney, right kidney, brain) following either vehicle administration (saline, *i.v.* control) or acute glycine administration (1.0 mmol/kg, *i.v.*) before and after infusion of NMDA antagonist, MK-801(75 mg/kg, *i.p.*) over a 45 min period in 14 week old WKY rats.

*p< 0.05, **p < 0.01 compared to respective control (n = 6 rats).

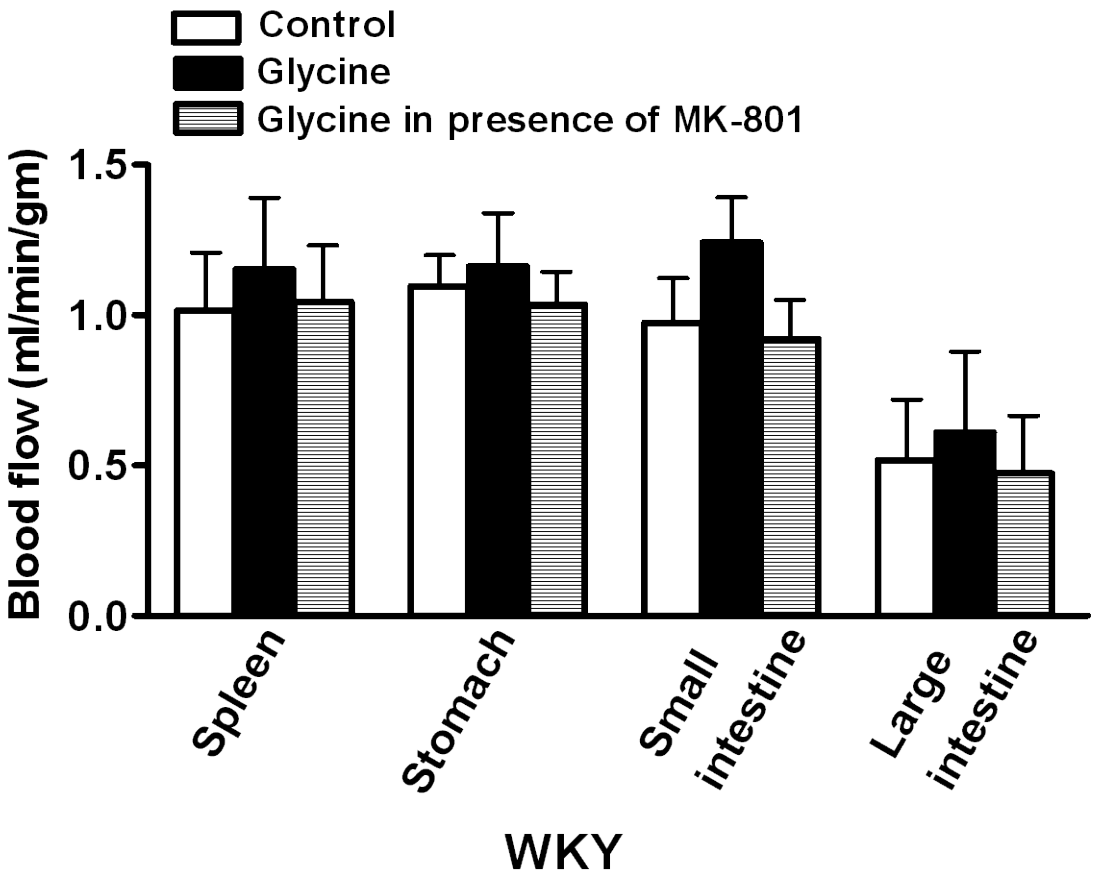

Figure 13 B. The bar diagrams give the level of blood flow (ml/min/gm) in various tissues/organs (spleen, stomach, small intestine, large intestine) following either acute vehicle administration (saline, *i.v.* control) or acute glycine administration (1.0 mmol/kg, *i.v.*) before and after administration of NMDA antagonist, MK-801(75 mg/kg, *i.p.*) over a 45 min period in 14 week old WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

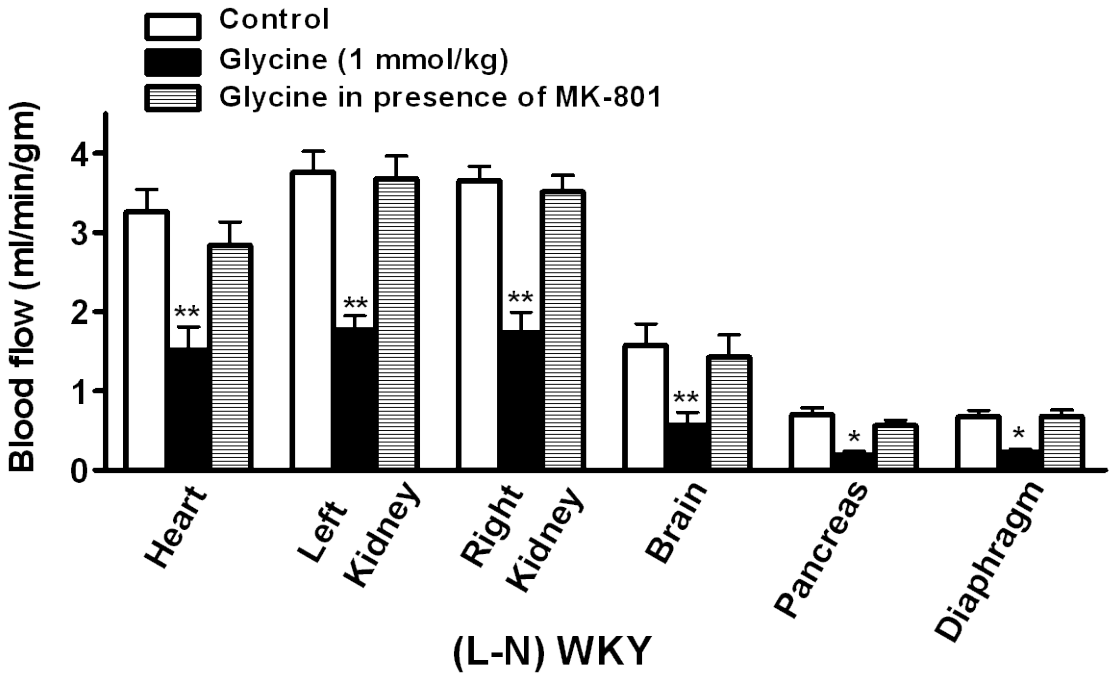

Figure 14 A. The bar diagram compares the blood flow (ml/min/gm) values in the following organs/tissues (Heart, left kidney, right kidney, brain, pancreas, diaphragm) following either vehicle administration (saline, *i.v.* control) or glycine administration (1.0 mmol/kg, *i.v.*) or glycine administration after MK-801 (75 mg/kg, *i.p.*) administration, over a period of 45 min in 14 week old chronic L-NAME (L-N) treated WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

*$p < 0.05$, **$p < 0.01$ compared to respective control.

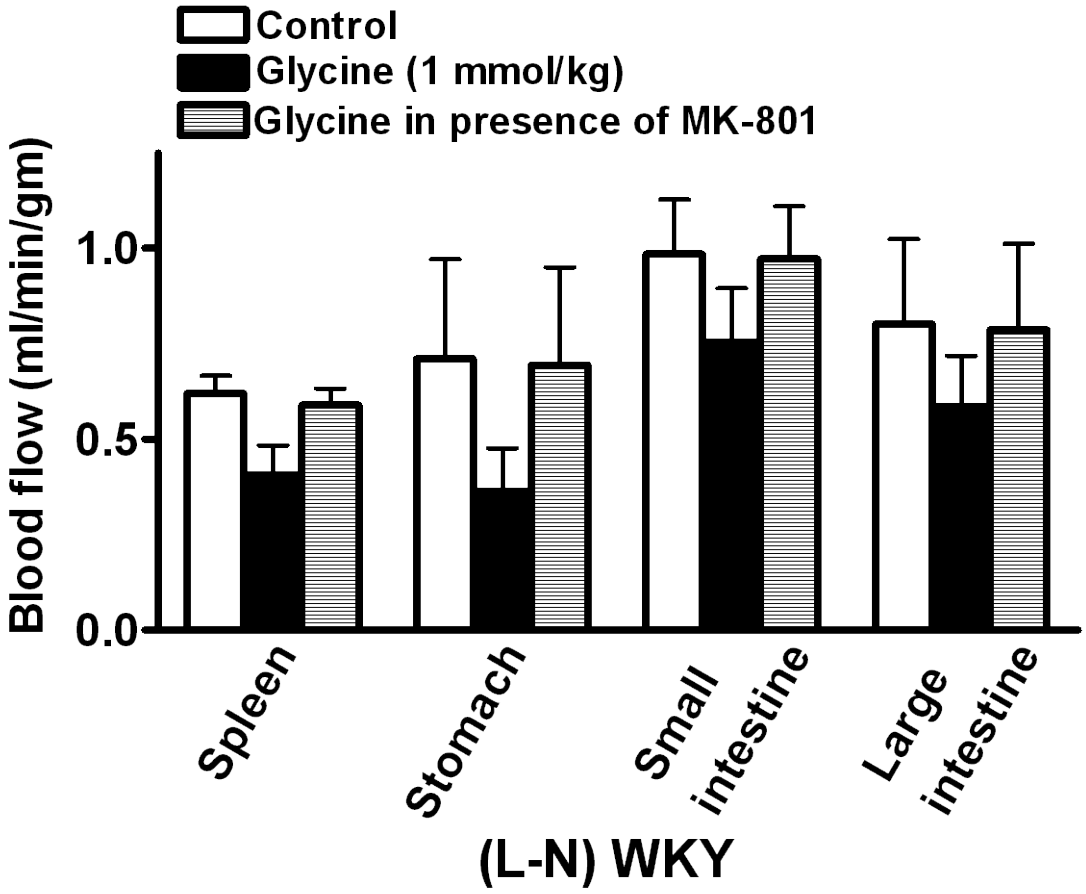

Figure 14 B. The bar diagram compares the blood flow (ml/min/gm) values in the indicated organs/tissues (spleen, stomach, small intestine and large intestine), following either vehicle administration (saline, *i.v.* control) or acute glycine administration (1.0 mmol/kg, *i.v.*) or glycine administration after slow infusion of MK-801 (75 mg/kg, *i.p.*) over a period of 45 min in 14 week old chronic L-NAME (L-N) treated WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

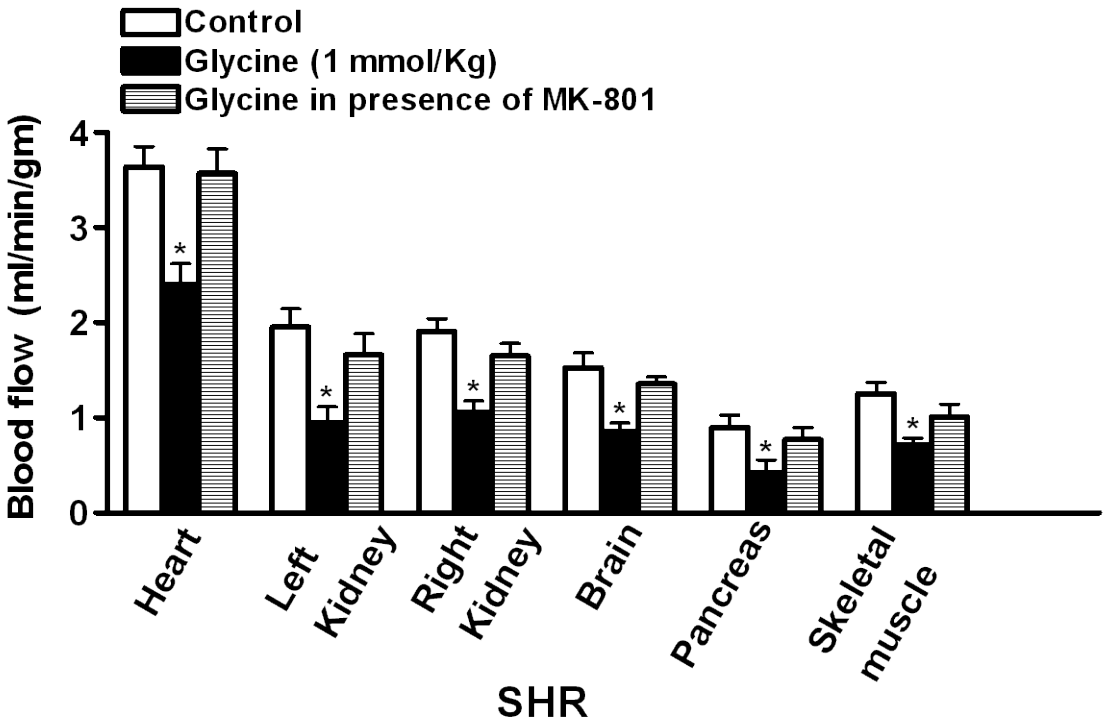

Figure 15 A. The bar diagram compares the blood flow (ml/min/gm) values in the indicated organs/tissues (heart, left kidney, right kidney, brain, pancreas, skeletal muscle), following either vehicle administration (saline, *i.v.* control) or acute glycine administration (1.0 mmol/kg, *i.v*) or glycine administration after slow infusion of NMDA antagonigt, MK-801 (75 mg/kg, *i.p.*), over a period of 45 min in 14 week old SHR. Each bar represents mean ± SEM value (n = 6 rats).

*$p < 0.05$ compared to respective control.

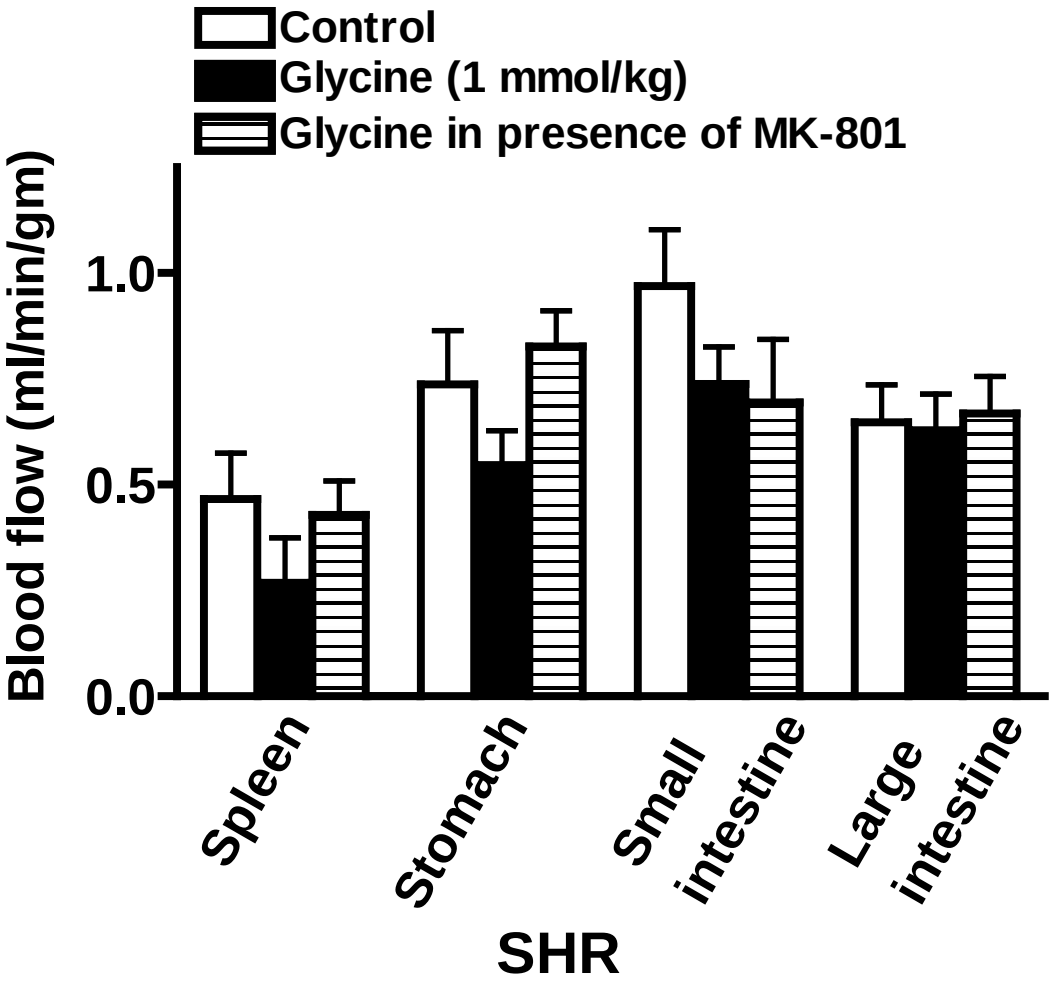

Figure 15 B. The bar diagram compares the blood flow (ml/min/gm) values in the indicated organs/tissues (spleen, stomach, small intestine and large intestine), following either vehicle administration (saline, *i.v.* control) or acute glycine administration (1.0 mmol/kg, *i.v.*) or glycine administration after slow infusion of NMDA antagonigt, MK-801 (75 mg/kg, *i.p.*), over a period of 45 min in 14 week old SHR. Each bar represents mean ± SEM value (n = 6 rats).

Table 3. Comparison of MAP (mmHg), heart rate (beats/min), cardiac output (ml/min) and total peripheral resistance (mmHg/ml/min) before and after acute *i.v.* glycine administration in 14 week old male WKY, L- NAME treated WKY and SHR strains.

Strain	Base line				Glycine			
	MAP	**HR**	**CO**	**TPR**	**MAP**	**HR**	**CO**	**TPR**
SD	112±2	366±5	108±5	1.05±0.2	79±6	378±4	125±6	0.5±0.07
WKY	116±5	366±9	106±5	1.09±0.08	76±8**	386±7	126±5**	0.6±0.04*
L-NAME treated WKY	158±3††	349±8	77±3††	2.05±0.16††	182±6*††	334±9	64±3*††	2.8±0.19**
SHR	183±7††	383±6†‡	83±2†	2.2±0.11††	210±6*††	360±6	63±3*††	3.3±0.24**

Each data is mean ± SEM values (n = 6 rats/group). *p<0.05 and **p<0.01 compared to control group.

†p<0.05 and ††p<0.01 compared to control group. ‡p<0.05 compared to L-NAME treated group.

Table 4. Comparison of blood flow (ml/min/gm) attained in various organs/tissues following either vehicle administration (saline, *i.v.* control group) or glycine (1.0 mmol/kg, *i.v.*) administration in 14 week old WKY, L-NAME treated WKY and SHR strains.

Strain	**WKY**		**L-NAME treated WKY**		**SHR**	
Tissue	**Control**	**Glycine**	**Control**	**Glycine**	**Control**	**Glycine**
	Blood flow (ml/min/g)					
Heart	2.897±0.279	4.647±0.301*	3.262±0.281	1.519±0.289**	3.369±0.213	2.406±0.215*
Left Kidney	3.641±0.128	6.860±0.125**	3.759±0.267	1.765± 0.183**	1.827±0.055†††‡‡‡	0.944±0.170*
Right Kidney	3.584±0.141	6.888±0.131**	3.656±0.180	1.733±0.259**	1.858±0.048†††‡‡‡	1.075±0.125*
Brain	1.144±0.106	1.946±0.253*	1.579±0.266	0.567±0.161**	1.527±0.154	0.841±0.094*
Pancreas	1.131±0.185	1.400±0.241	0.701±0.082	0.196±0.042*	0.900±0.129	0.426±0.132*
Diaphragm	0.192±0.085	0.256±0.124	0.675±0.80	0.226±0.04 *	0.332±0.057	0.289±0.042
Skeletal muscle	1.085±0.149	1.378±0.182	1.235±0.148	0.948±0.101	1.256±0.115	0.719±0.068*
Spleen	0.620±0.046	0.407±0.077	1.016±0.193	1.153±0.237	0.466±0.109‡	0.268±0.107
Stomach	0.711±0.261	0.364±0.112	1.097±0.102	1.163±0.176	0.737±0.127	0.547±0.081
Small intestine	0.985±0.142	0.755±0.140	0.975±0.148	1.241±0.151	0.970±0.133	0.738±0.087
Large intestine	0.802±0.222	0.585±0.133	0.518±0.202	0.611±0.268	0.647±0.089	0.629±0.085

Each data is mean ± SEM value (n = 6 rats/group). *p<0.05, **p<0.01 compared to respective control group. †††p<0.001 compared to respective control WKY group, ‡p<0.05, ‡‡‡p<0.001 compared to respective L-NAME treated WKY group.

Table 5. Comparison of regional vascular resistance (mmHg/ml/min/gm) attained in various organs/tissues following either vehicle administration (saline, *i.v.* control group) or glycine (1.0 mmol/kg, *i.v.*) administration in 14 week old male WKY, L-NAME treated WKY and SHR strain.

Tissue	Regional vascular resistance (mm Hg/ml/min/gm)					
	WKY Control (Saline)	WKY Control (Glycine)	WKY(L-NAME) (Saline)	WKY(L-NAME) (Glycine)	SHR (Saline)	SHR (Glycine)
Heart	41.42±3.305	16.35±3.5***	48.43±6.5	119.81±22*	54.31±3.21†	87.50±3.99***‡
Left Kidney	32.77±2.39	11.07±3.9***	42.03±2.6	103.11±13.5**	100.16±9.69†††11	224.45±15.7***‡
Right Kidney	33.14±4.14	11.04±1.2***	43.21±3.4	105.02±11.3**	98.49±5.14††† 11	195.34±14.9***‡
Brain	128.31±6.3	39.05±11.3***	100±8.2†	320.98±40.7***	119.84±11.2	249.70±16.7***‡
Pancreas	102.65±18.9	54.28±14.2	225.39±19.3††	928.57±15.6***	203.33±15.4††	292.07±18.9**‡‡
Diaphragm	604.16±34.4	296.87±30.9***	233.72±19.1†††	805.30±16.4***	551.20±15.311	726.64±23.5***‡
Skeletal muscle	106.91±24.8	55.15±6.5	141.32±12.4	145.60±30.1	145.7±15.3	292.07±14.9***‡‡
Spleen	187.09±18.5	186.73±24.6	254.83±16.1†	179.13±25.9	392.7±36.2†††1	453.51±38.3
Stomach	163.38±16.1	208.79±15.2	222.22±22.5	156.49±28.9	248.3±40.23	383.91±61.3
Small intestine	117.76±14.4	100.66±16.7	162.05±15.2	146.65±26.8	188.65±15.42††	284.55±46.9
Large intestine	114.63±14.9	129.91±12.6	305.01±33.5†††	297.87±31.4	282.84±35.85	333.86±17.4

Each data point is mean ± SEM of 6 rats. *p<0.05, **p<0.01 and ***p<0.001 compared to vehicle treatment in the same group. †p<0.05 and ††p<0.01 compared to respective data of WKY group. ‡p<0.05, ‡‡ p<0.01, ‡‡‡ p<0.001, L-NAME vs. SHR group. ^{1}p<0.05, ^{11}p<0.01 compared to L-NAME treated WKY group.

4.8. *In vivo* studies

4.8.1. Acute hemodynamic effect of glycine in presence of NMDA blockers, MK-801/memantine

Pooled data from several experiments revealed that, acute glycine (1.0 mmol/kg) administration evoked a fall in MAP in normotensive WKY rats (Figure 16). This fall in MAP (79 ± 7) was statistically significant ($P < 0.05$) compared to the baseline MAP (114 ± 6) in normotensive WKY rats (Figure 16). This decrease in MAP evoked by acute glycine administration was abolished by pretreatment with NMDA antagonist either MK-801 (75 mg/kg) or memantine (50 mg/kg) in these group of rats (Figure 16). Conversely, in L-NAME treated hypertensive WKY rats, acute glycine (1.0 mmol/kg) administration evoked a significant increase ($P < 0.05$) in pressor response (180 ± 3 vs. 155 ± 4) compared to vehicle administration in the same group of rats (Figure17). This increase in MAP was attenuated and comparable to control value in presence of MK-801 (75 mg/kg) or memantine (50 mg/kg) in this group of hypertensive rats (Figure 17). In SHR strain glycine (1.0 mmol/kg) administration also evoked an increase in MAP (204 ± 5 vs. 177 ± 8) compared to vehicle administration in the same group and this pressor response was abolished in the presence of the MK-801 (75 mg/kg) or memantine (50 mg/kg), when glycine was administered (Figure 18). From this data it is clearly evident that acute glycine administration evokes a depressor response in normotensive and a pressor response in hypertensive rat models and these responses were abolished when the rats were pretreatment with NMDA antagonist MK-801 or memantine.

4.8.2. Acute hemodynamic effect of glycine in presence of glycine transporter inhibitor, sarcosine

Glycine (1.0 mmol/kg) evoked a fall in MAP that was increased further in the presence of sarcosine, a glycine transporter inhibitor in normotensive WKY rats (Figure 16). Pretreatment with sarcosine (100 mg/kg) enhanced the fall in MAP significantly ($P < 0.05$) compared to fall in MAP following glycine administration alone (64 ± 3 vs. 79 ± 7) in normotenive WKY rats (Figure 16). The glycine evoked fall in MAP in the absence of sarcosine was (79 ± 7 vs. 114 ± 6, $P < 0.01$), whereas in the presence of sarcosine this response was onefold higher (64 ± 3 vs. 114 ± 6, $P < 0.001$) compared to vehicle administration in normotensive WKY rats (Figure 16). Glycine (1.0 mmol/kg) evoked a pressor response (180 ± 3 vs. 155 ± 4, $P < 0.05$) compared to vehicle administration in the L-NAME pretreated WKY rats (Figure 17). This pressor response was increased further (215 ± 4 vs. 180 ± 3, $P < 0.05$) when glycine was administered in the presence of sarcosine (100 mg/kg) in this group of rats (Figure 17). Glycine administration in presense of sarcosine was significantly higher (215 ± 4 vs. 155 ± 4, $P < 0.001$) compared to vehicle administration (Figure 17). In the SHR strain, acute glycine administration increased the MAP (204 ± 5 vs. 177 ± 8, $P < 0.05$) compared to vehicle administration (Figure 18). When glycine was administered acutely in presence of sarcosine (100 mg/kg), this pressor response was significantly elevated ($P < 0.05$) compared to (231 ± 4 vs. 204 ± 5) glycine administration alone in the SHR strain (Figure 18). We also compared the acute glycine administration in presense of sarcosine (100 mg/kg) and the pressor response to glycine was found to be significantly higher ($P < 0.01$) compared to (231 ± 4 vs. 177 ± 8) vehicle administration in this group of rats (Figure 18).

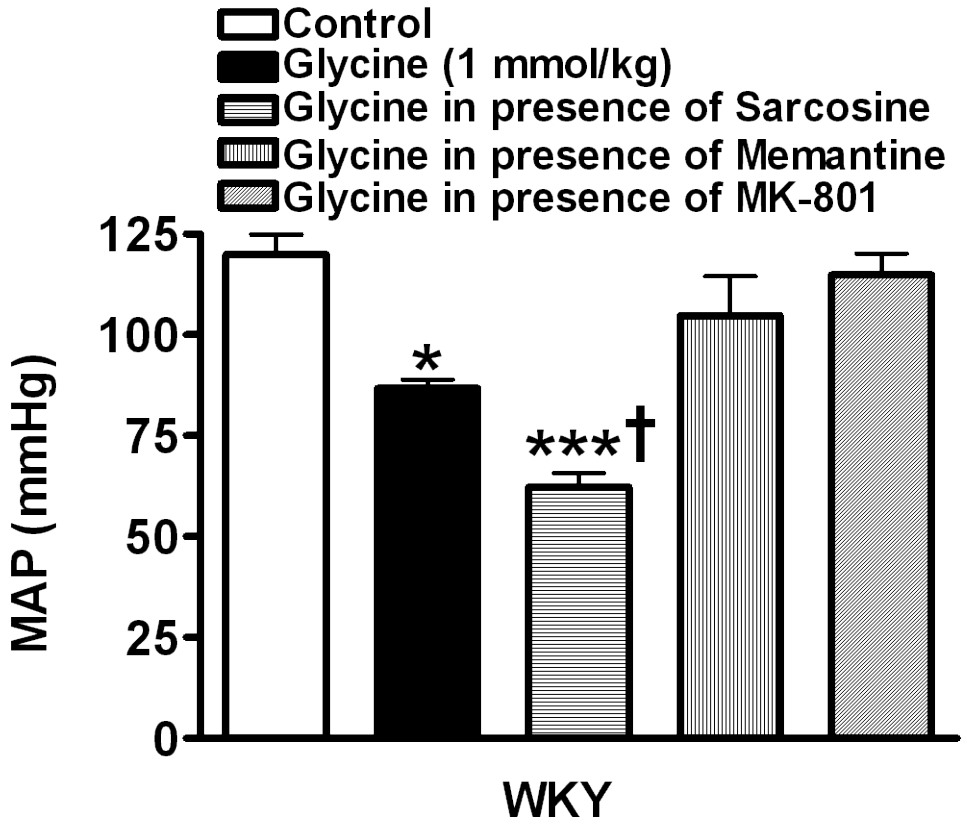

Figure 16. The bar diagram compares the MAP (mmHg) following vehicle (saline, *i.v.* control) administration or acute glycine administration (1.0 mmol/kg, *i.v*), either in the presence or absence of sarcosine (100 mg/kg, *i.p.*) in 14 week old male WKY rats. This diagram also compares the MAP (mmHg) following vehicle (saline, *i.v.* control) administration and acute glycine administration after slow infusion of NMDA antagonist memantine (50 mg/kg, *i.p.*) and MK-801 (75 mg/kg, *i.p*) in 14 week old male WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

*$p < 0.05$, ***$p < 0.001$ compared to respective control.

$^{\dagger}p < 0.05$ compared to glycine treatment.

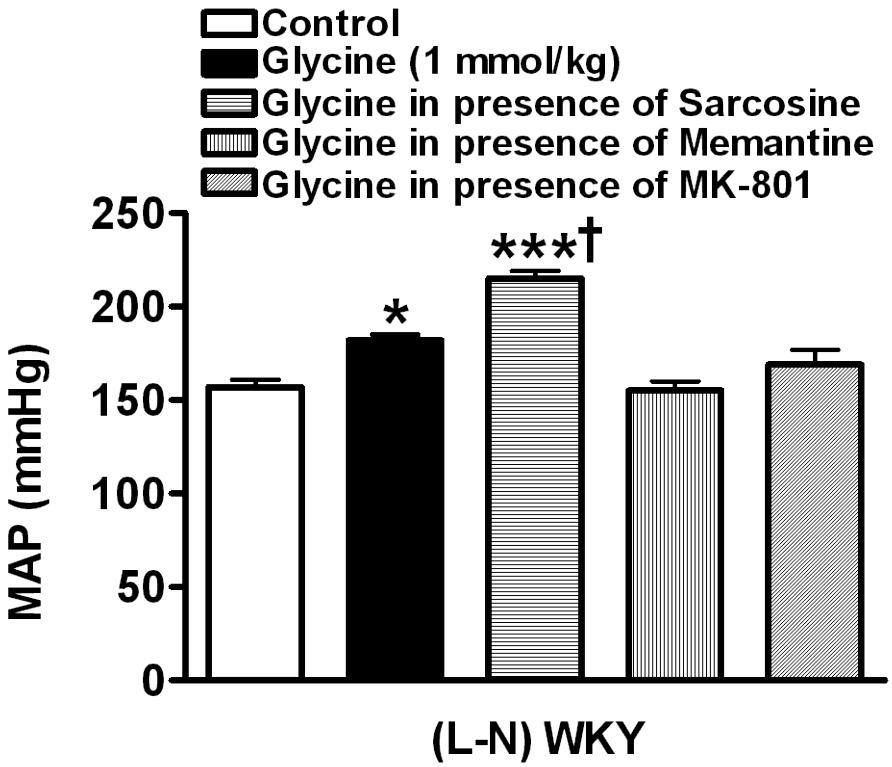

Figure 17. The bar diagram compares the MAP (mmHg) following vehicle (saline, *i.v.* control) administration or acute glycine administration (1.0 mmol/kg, *i.v*), either in the presence or absence of sarcosine (100 mg/kg, *i.p.*) in 14 week old male L-NAME treated (L-N) WKY rats. This diagram also compares the MAP (mmHg) following vehicle (saline, *i.v.* control) administration and acute glycine administration after slow infusion of NMDA antagonist memantine (50 mg/kg, *i.p.*) and MK-801 (75 mg/kg, *i.p*) in 14 week old male L-NAME treated WKY rats. Each bar represents mean ± SEM value (n = 6 rats).

*$p < 0.05$, ***$p < 0.001$ compared to respective control.

$^{\dagger}p < 0.05$ compared to glycine treatment.

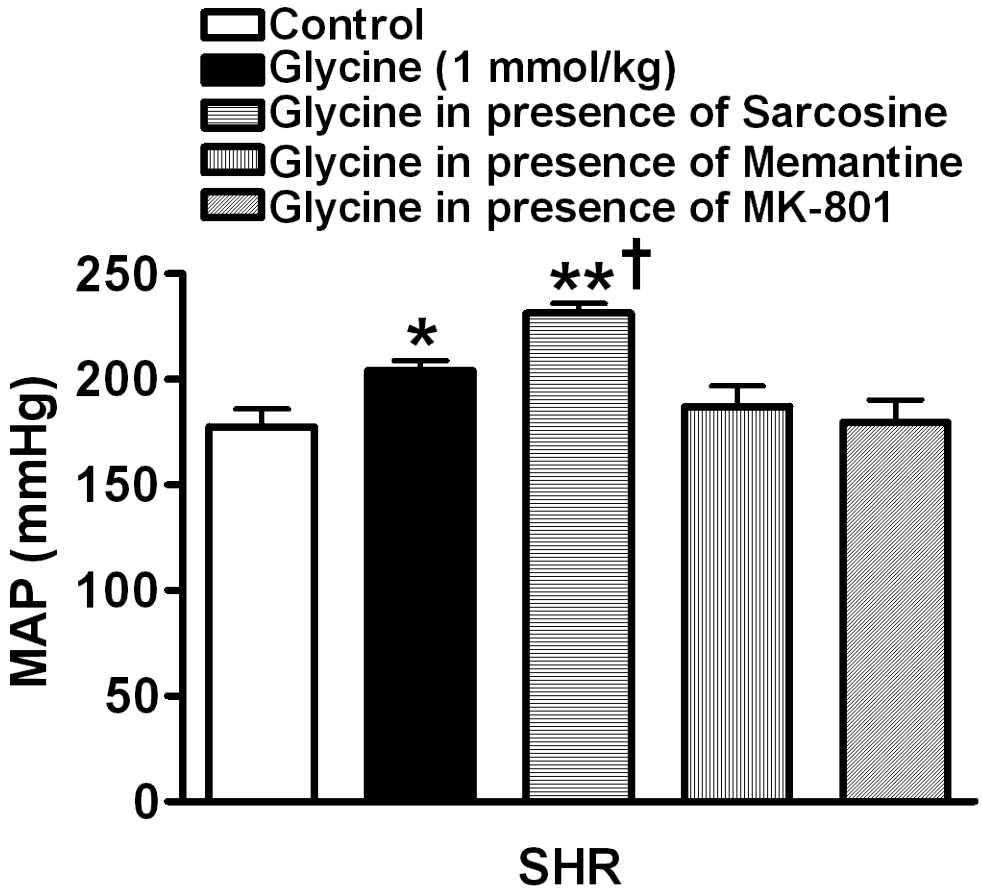

Figure 18. The bar diagram compares the MAP (mmHg) following vehicle (saline, *i.v.* control) administration or acute glycine administration (1.0 mmol/kg, *i.v*), either in the presence or absence of sarcosine (100 mg/kg, *i.p.*) in 14 week old SHR strains. This diagram also compares the MAP (mmHg) following vehicle (saline, *i.v.* control) administration and acute glycine administration after slow infusion of NMDA antagonist memantine (50 mg/kg, *i.p.*) and MK-801 (75 mg/kg, *i.p*) in 14 week old SHR strains. Each bar represents mean ± SEM value (n = 6 rats).

*$p < 0.05$, ***$p < 0.001$ compared to respective control.

$^{\dagger}p < 0.05$ compared to glycine treatment.

4.9. *In vitro* studies (using rat aortic rings)

We conducted several experiments using thoracic aorta isolated from both normotensive WKY rats and SHR. Pooled data from these experiments revealed that glycine at different pharmacological concentrations evoked opposite responses in these normotensive and hypertensive rats. The results obtained from these *in vitro* studies were pretty consistent with our *in vivo* results. Glycine produced a vasodilator response in the aortic rings isolated from normotensive WKY rats (Figure 19). On the contrary glycine produced a contractile response in aortic rings isolated from SHR strain (Figure 22).

4.9.1. Vasodilator response of glycine in normotensive WKY rats

In phenylephrine (PE) constricted aortic rings glycine evoked (0.5 to 3.0 mmol/L) an endothelium and concentration-dependent vasodilatation (Figure 19). This vasodilator effect to glycine was abolished in endothelium denuded aortic ring preparation (Figure 20). The response to glycine was found to be with much lower efficacy compare to the response elicited by ACh in the aortic rings isolated from normotensive WKY rats (Figure 21). The maximal response (E_{max}) to glycine was (40 ± 3 %) at 1.0 mmol/L and the effective median concentration (EC_{50}) value calculated was (376.7 ± 158.7 μmol/L) in endothelium-intact aortic rings (Figure 20 and Figure 29).

4.9.2. Vasoconstrictor response of glycine in SHR

Glycine (0.5 to 3.0 mmol/L) evoked a dose dependent contractile response in PE constricted aortic rings isolated from SHR (Figure 22). This result supports our *in vivo* observations where glycine evoked an increase in MAP by increasing peripheral vascular

resistance and decreasing organ blood flow to several key organs/tissues (Table 3, Table 4 and Table 5).

4.9.3. Increase in basal tone by glycine in normotensive and hypertensive rat models

We demonstrated the vascular effect of glycine in isolated aotic rings in absence of contractile agonist. Interestingly, glycine in the absence of PE increased the basal tone by increasing the basal preload tension (Max 1.4 g) in all aortic ring preparations from normotensive WKY rats as well as hypertensive SHR (Figure 23 and Figure 24). This increase in the basal tone was due to the activation of vascular NMDA receptors.

4.9.4. Inhibition of glycine mediated response in the presence of either L-NAME/MK-801

Aortic rings preincubated with NMDA receptor antagonist MK-801 (10 μmol/L) for 30 minutes, completely inhibited glycine induced increase in basal tone in normotensive WKY rats and SHR strains (Figure 25, Figure 26 and Figure 27). In another set of experiments, pretreatment with L-NAME (100 μmol/L) for 30 minutes, abolished the vasodilator response to glycine in PE constricted aortic rings, isolated from normotensive WKY rats (Figure 28 and Figure 29). Increase in the basal tone evoked by glycine was compared with contractile agonists PE and potassium chloride (KCl). Although glycine, PE and KCl increased the basal tone in aortinc rings isolated from normotenive and hypertensive rats, pretreatment with NMDA antagonist MK-801 only inhibited the increased basal tone evoked by glycine but not to PE or KCl (Figure 26). Glycine evoked a dose dependent vasodilatation in aortic rings isolated from

normotensive WKY rats and this response was significantly inhibited when aortic rings are pretreated with NMDA antagonist MK-801 for 30 minutes (Figure 30 and Figure 31). In PE constricted aortic rings isolated from SHR produced a further contractile response to glycine and this was blocked by pretreatment with NMDA antagonist MK-801 (Figure 32, Figure 33). Both the decrease and increase in MAP was blocked by pretreatment with NMDA antagonist MK-801 (Figure 8 A, B, C and Figure 12 A, B, C). Similarly, in aortic rings pretreated with NOS inhibitor L-NAME or in endothelium denuded preparation, we did not observe glycine mediated vasodilatation in these vessels isolated from normotensine WKY rats (Figure 20 and Figure 28).

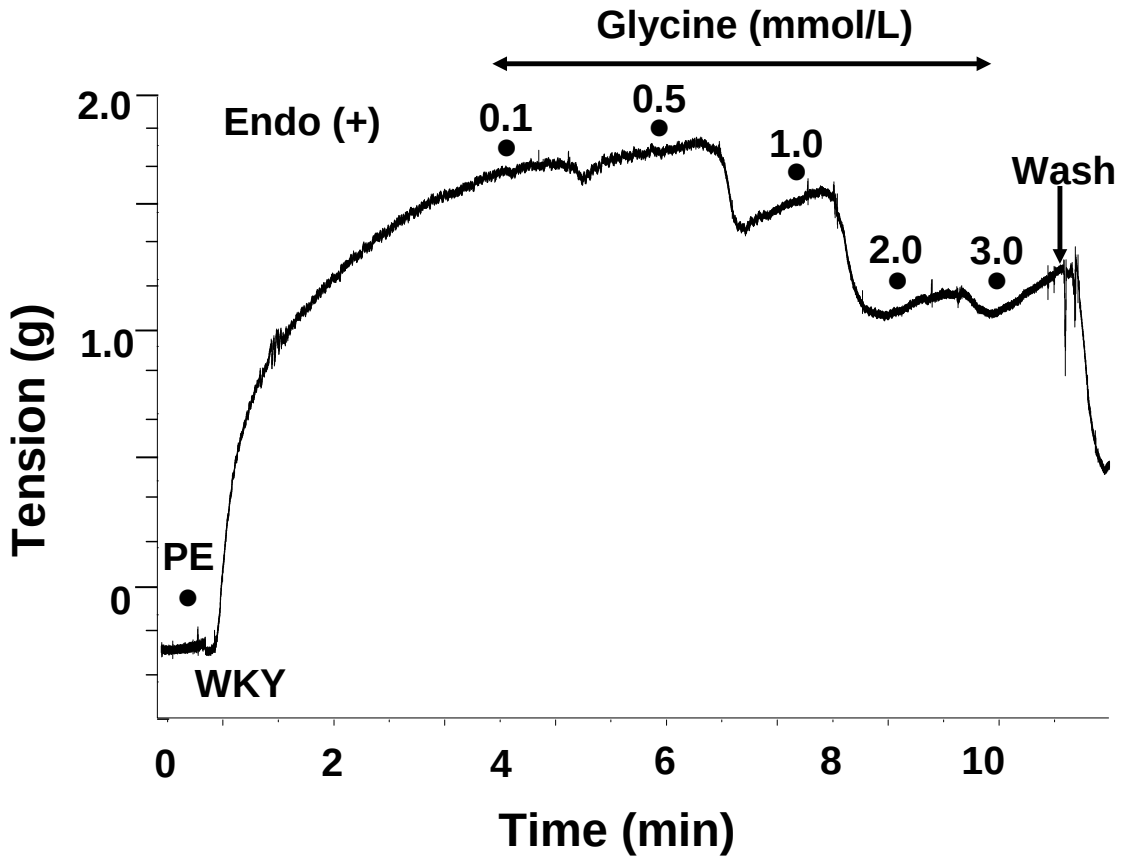

Figure 19. A typical experiment that shows concentration dependent (0.1 to 3.0 mmol/L) vasodilatation evoked by *in vitro* addition of glycine (0.1 to 3.0 mmol/L) in (PE-1 μmol/L) constricted endothelium-intact rat aortic rings isolated from a WKY rat. Similar responses were reproduced in 6 WKY rats (n = 6).

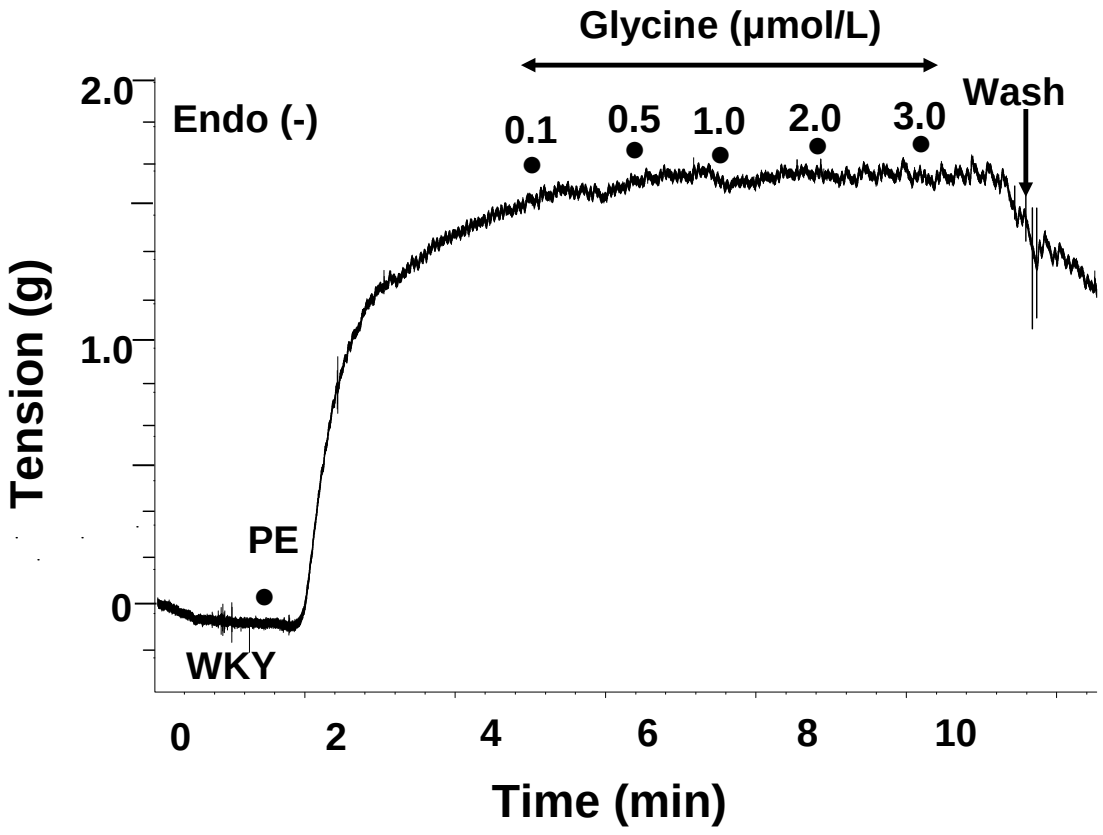

Figure 20. A typical experiment that demonstrates lack of a concentration dependent vasodilator response, to increasing concentrations of glycine (0.1 to 3.0 mmol/L), in PE (1 µmol/L) constricted (but endothelium-denuded) rat aortic rings isolated from WKY rat. Similar results were seen in 6 WKY rats (n = 6).

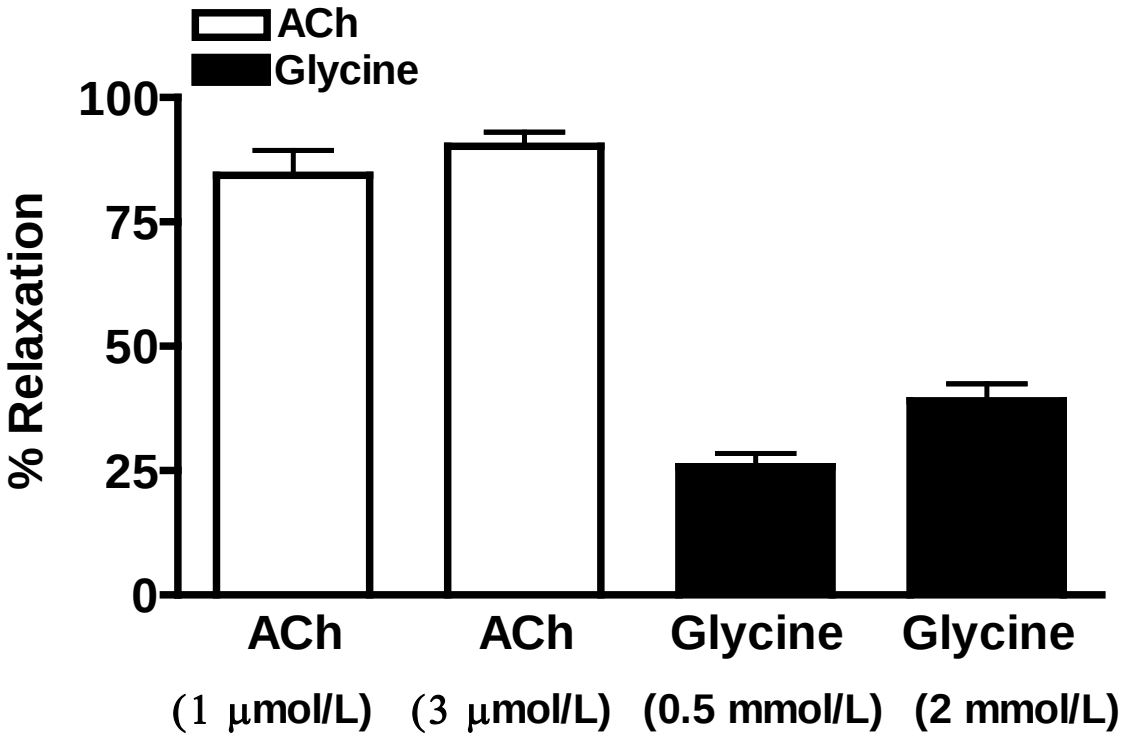

Figure 21. Comparison of vasodilator responses to ACh and glycine in endothelium-intact PE (1 μmol/L) constricted rat aortic rings isolated from WKY rats. Each bar is a mean ± SEM value (n = 6).

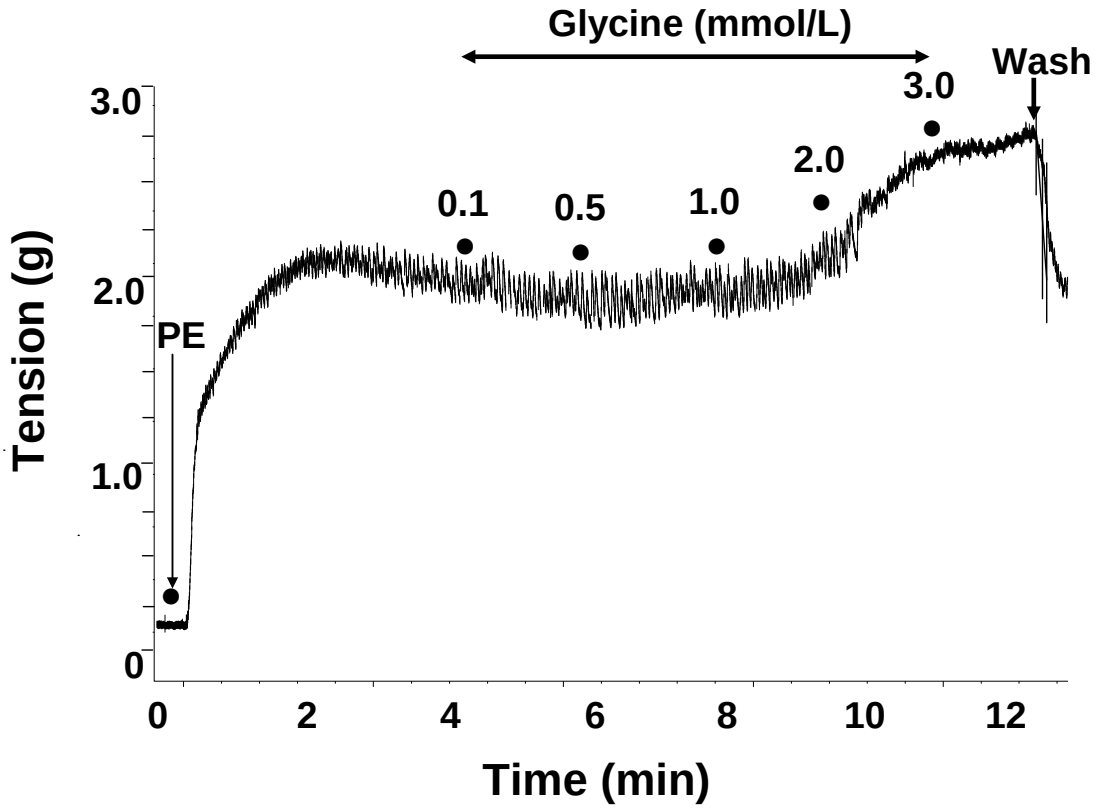

Figure 22. A typical experiment that demonstrates the *in vitro* addition of glycine (0.1 to 3.0 mmol/L), in a PE (1 μmol/L) constricted aortic ring isolated from SHR strain.

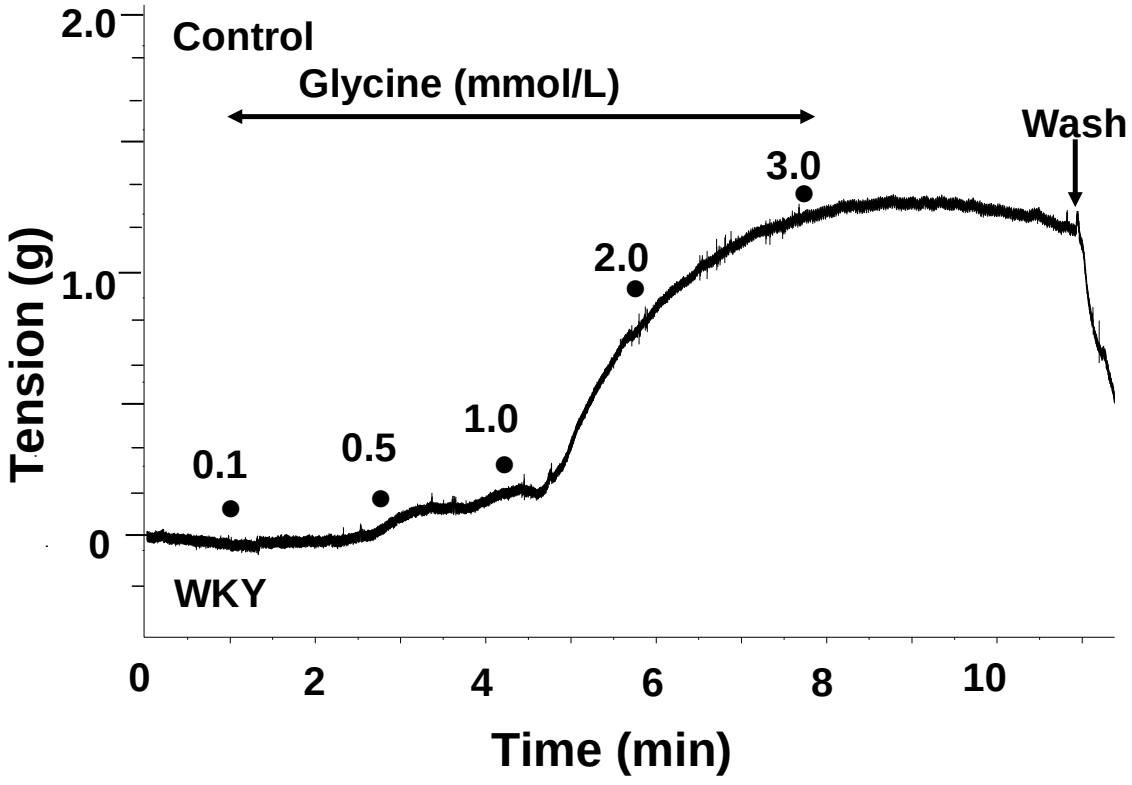

Figure 23. A typical experiment that demonstrates the increase in basal vascular tone attained following cumulative addition of glycine (0.1 to 3.0 mmol/L) *in vitro* to rat aortic rings after its isolation from a 14 week old male WKY rats. Similar data were reproduced in 6 rats (n = 6).

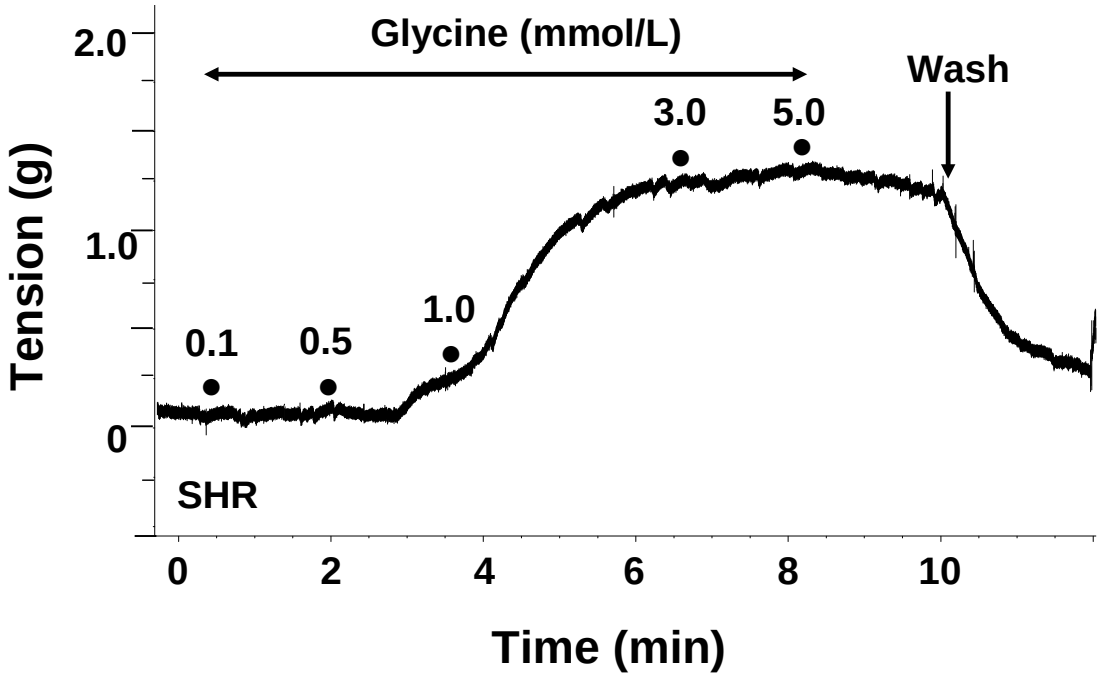

Figure 24. A typical representative experiment that shows glycine (0.1 to 3.0 mmol/L) evokes concentration-dependent (0.1 to 3.0 mmol/L) increase in basal tone in aortic rings isolated from SHR strain. This was seen in at least 8 rings isolated from 5 rats.

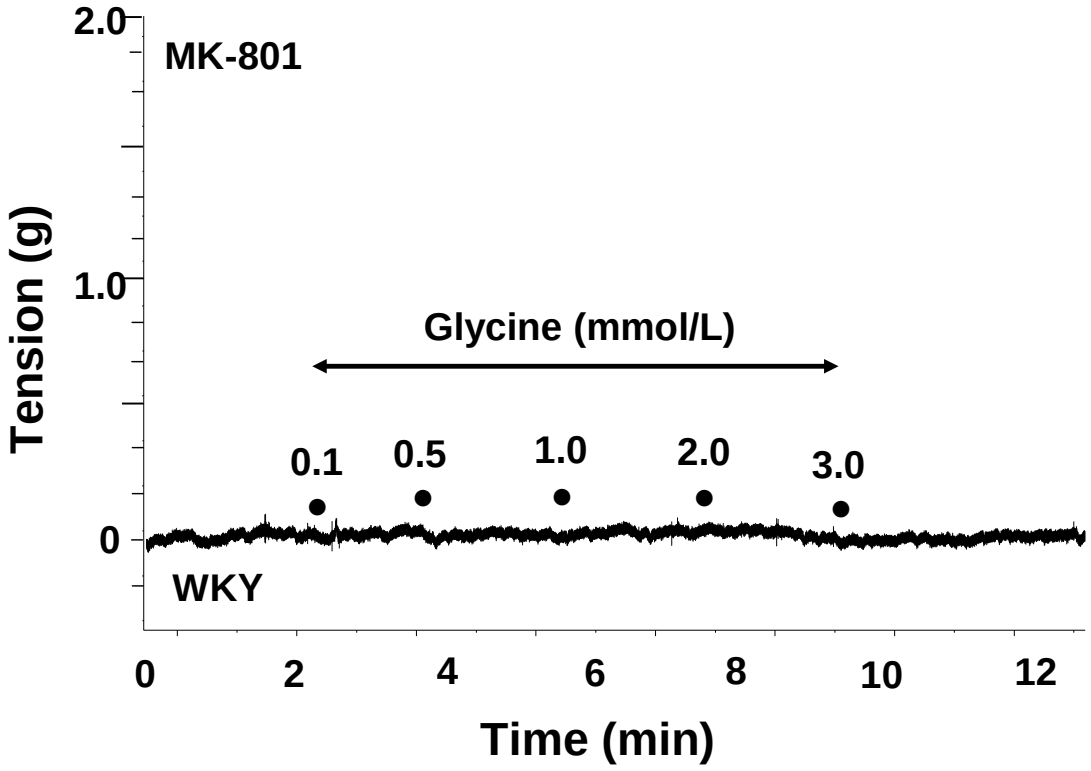

Figure 25. A typical experiment that demonstrates lack of increased basal tone following addition of increasing concentrations of glycine (0.1 to 3.0 mmol/L) in rat aortic rings of WKY rat maintained in the presence of NMDA antagonist, MK-801 (10 μmol/L). This was seen in at least 8 rings isolated from 5 rats irrespective of whether the endothelium was present or absent.

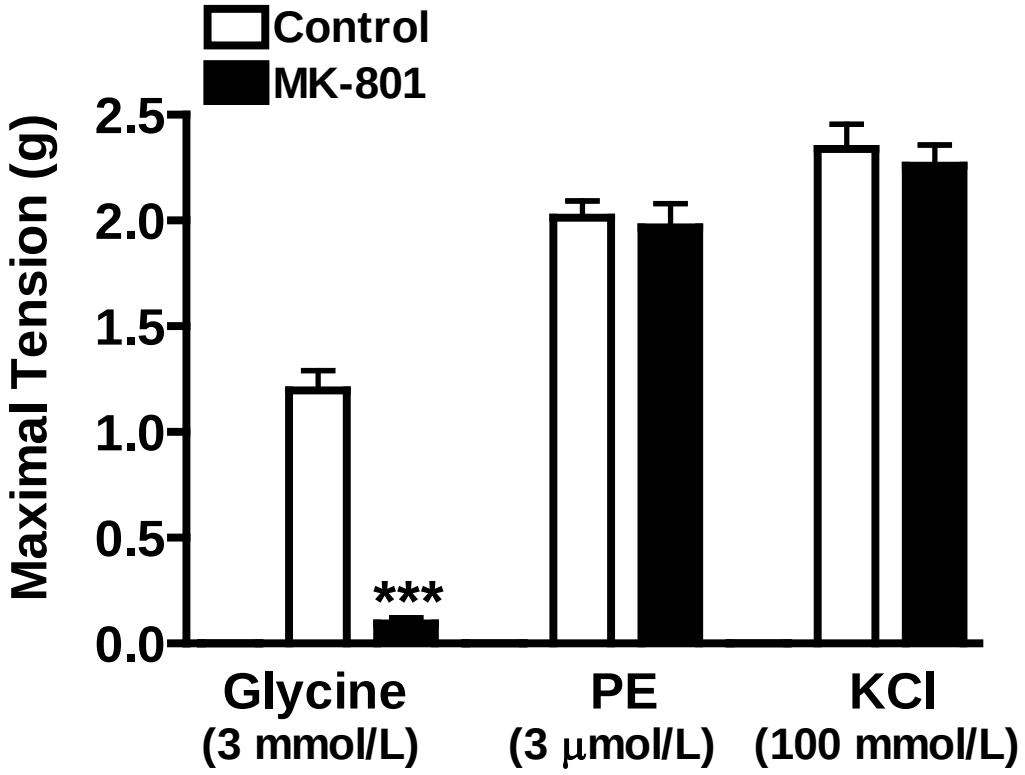

Figure 26. The bar diagrams compare the maximal vasoconstrictor/tension responses generated by maximal concentrations of either glycine (3.0 mmol/L) or PE (3 μmol/L) or KCl (100 mmol/L) in WKY rat aortic rings with intact endothelium, in the presence and absence of NMDA antagonist MK-801 (10 μmol/L). Each data is mean ± SEM value (n = 6).

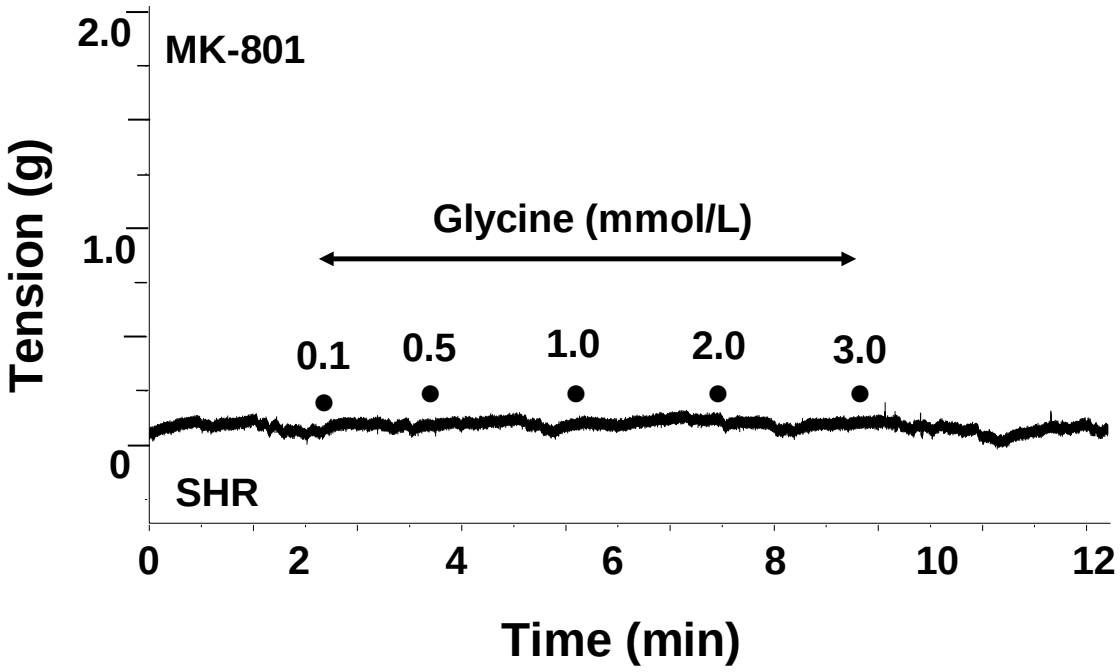

Figure 27. A typical experiment that demonstrates lack of increased basal tone following addition of increasing concentrations of glycine (0.1 to 3.0 mmol/L) in rat aortic rings of SHR maintained in the presence of NMDA antagonist, MK-801 (10 μmol/L). This was seen in at least 8 rings isolated from 5 rats.

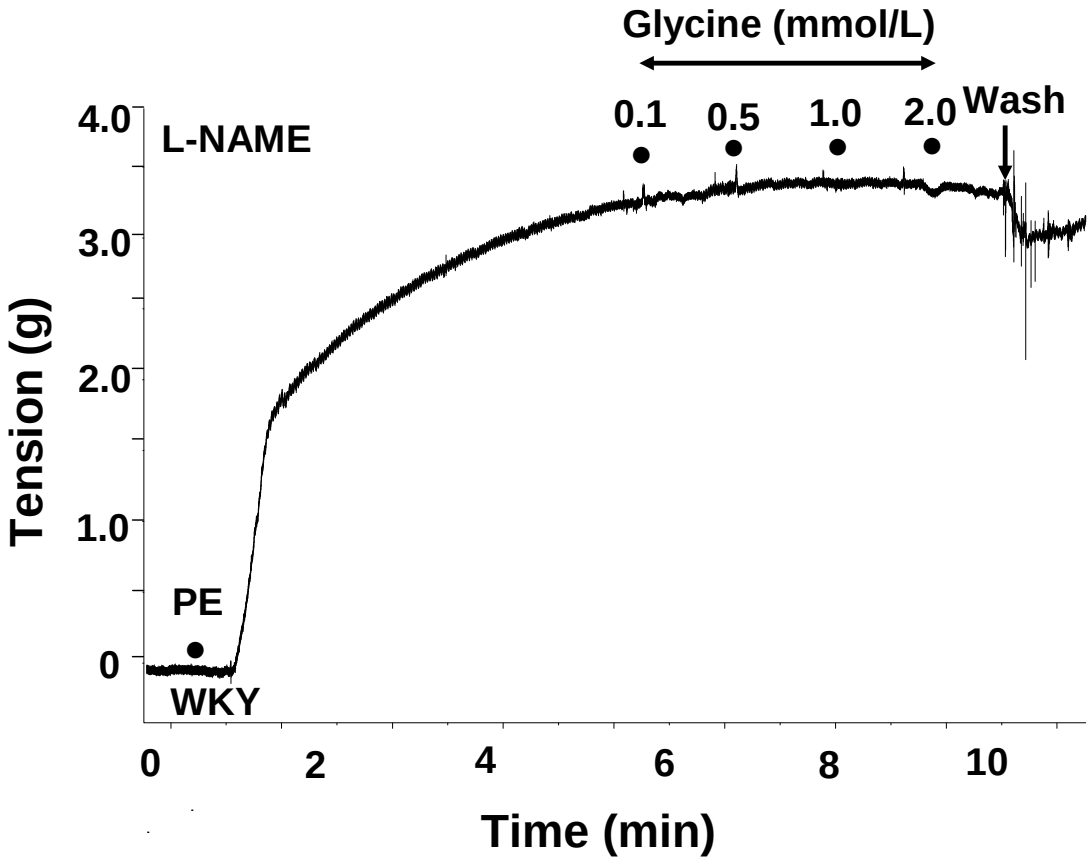

Figure 28. A typical experiment that demonstrates lack of vasodilator responses to glycine (0.1 to 3.0 mmol/L) in PE (1 μmol/L) constricted rat aortic rings in the presence of NOS inhibitor, L-NAME (100 μmol/L) in the incubated medium.

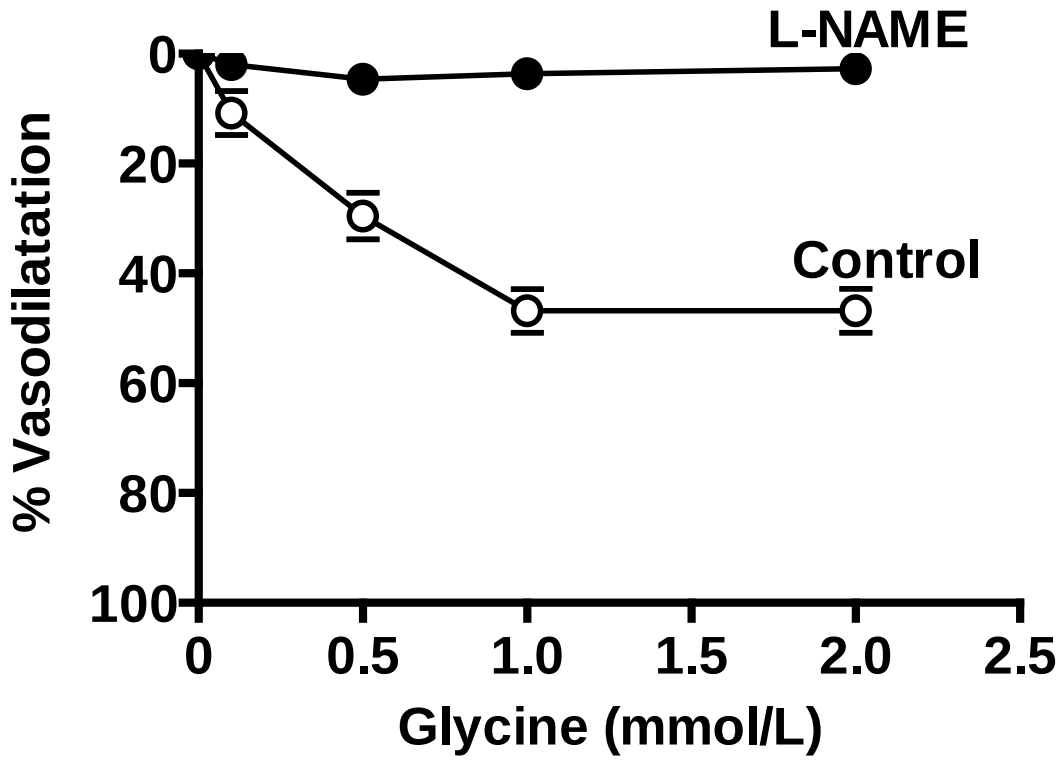

Figure 29. Concentration response curve compares the vasodilatation to glycine (0.1 to 2.0 mmol/L) in presence and absence of NOS inhibitor L-NAME (100 μmol/L), in aortic rings isolated from normotensive WKY rat.

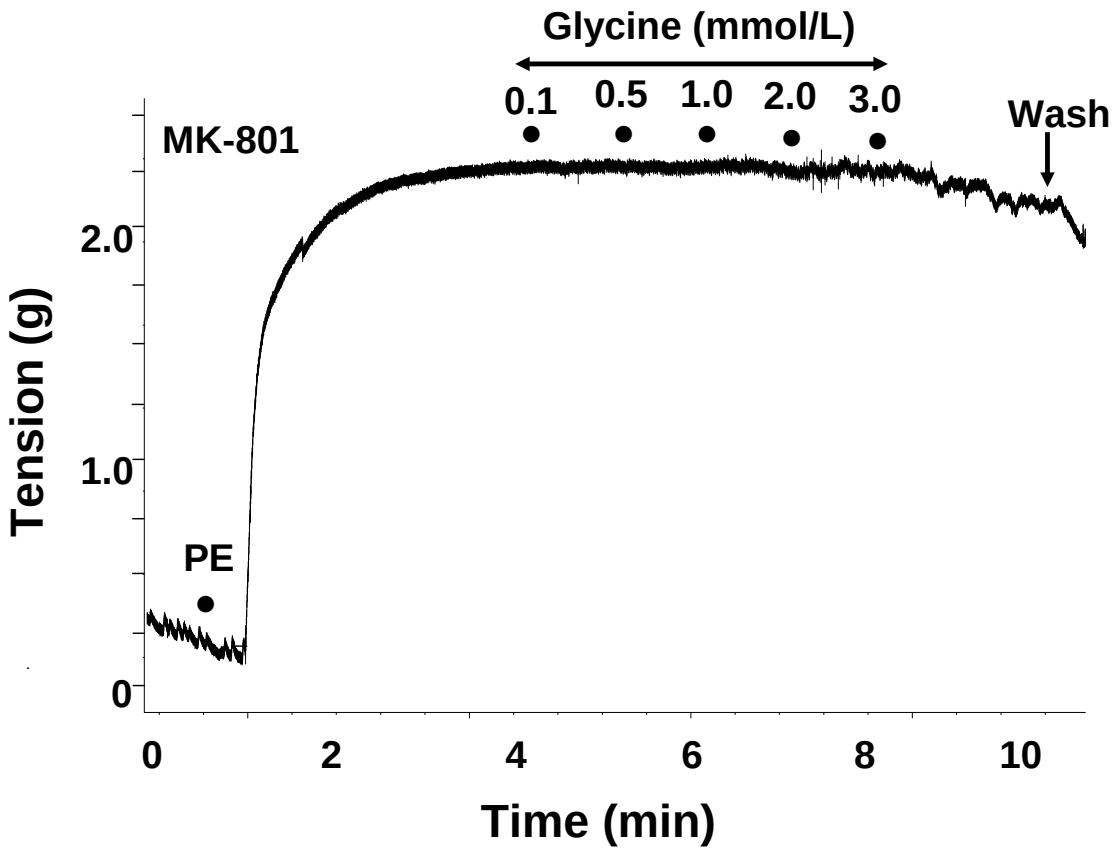

Figure 30. A typical experiment that demonstrates lack of vasodilatation to increasing concentrations of glycine (0.1 to 3.0 mmol/L) in PE (1 μmol/L) constricted rat aortic rings of WKY rats, when NMDA antagonist, MK-801 (10 μmol/L) is kept in the incubation medium.

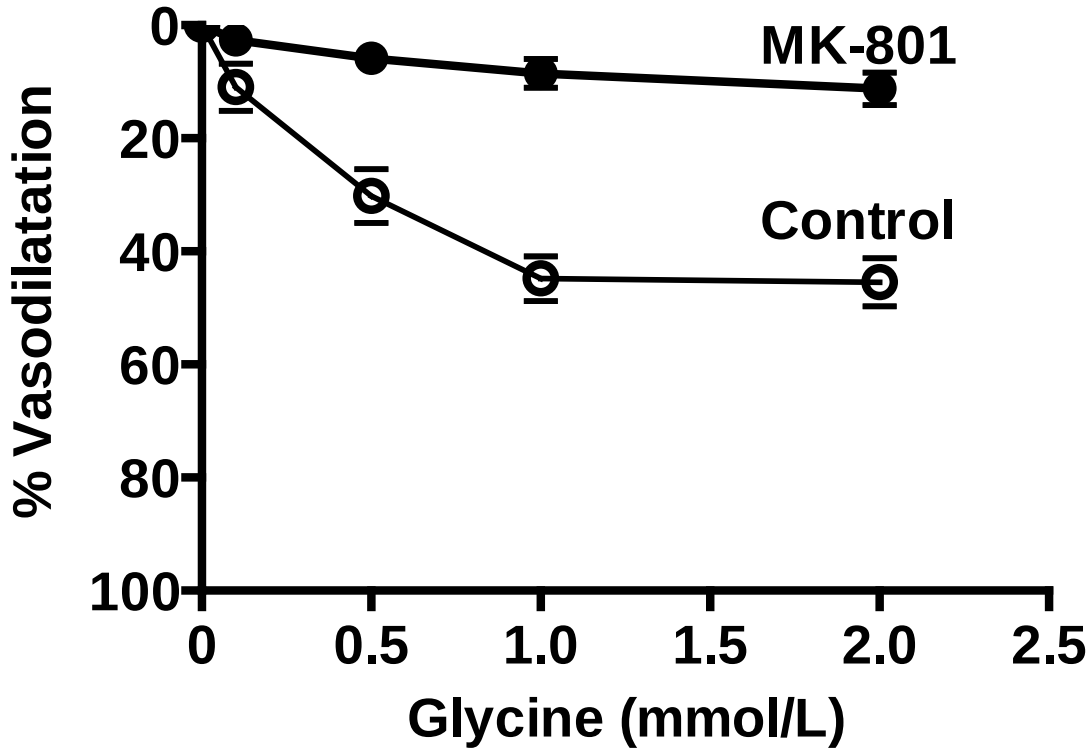

Figure 31. Concentration response curves to glycine (0.1 to 2.0 mmol/L) determined in PE (1 μmol/L) constricted rat aortic rings with intact endothelium either in the presence of NMDA antagonist, MK-801 (10 μmol/L), or absence (control) of MK-801, maintained in the incubation medium. Each data pint is mean ± SEM value (n = 6 rats).

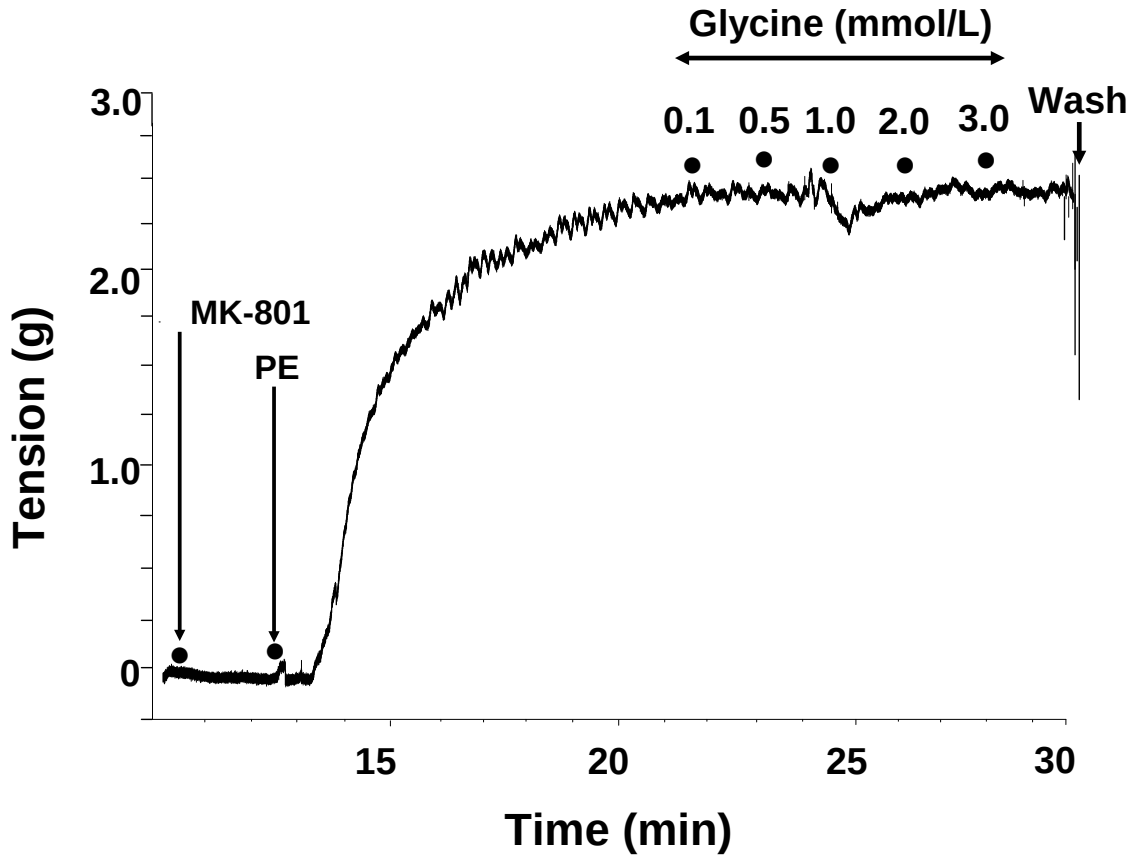

Figure 32. A typical experiment that shows glycine (0.1 to 3.0 mmol/L) evoked vasodilation is not present in PE (1 μmol/L) constricted SHR aortic ring, when NMDA antagonist, MK-801(10 μmol/L) is added in the incubation medium.

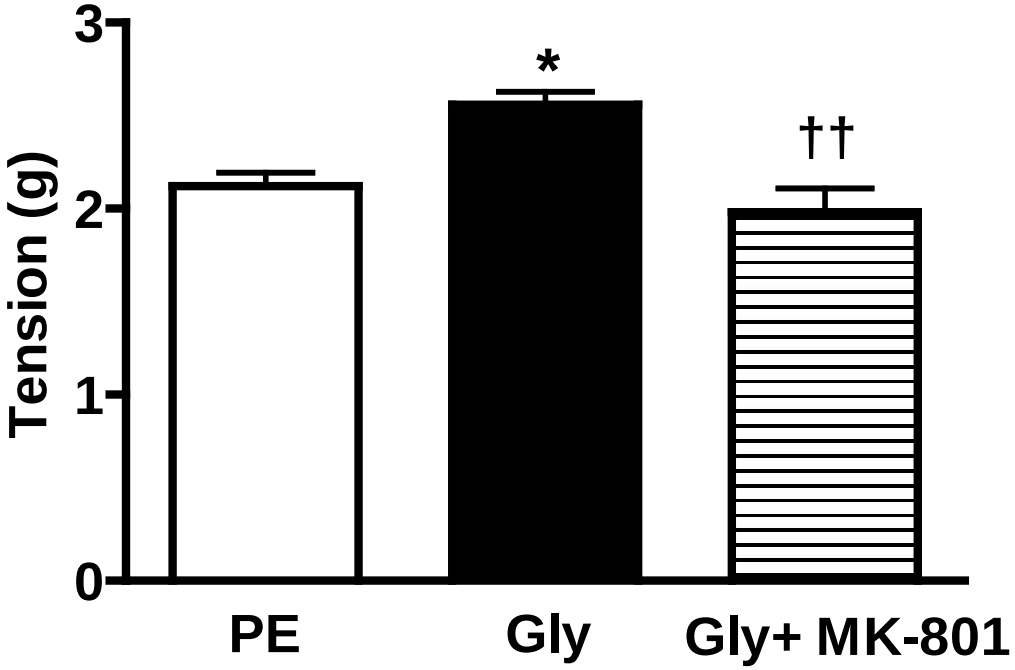

Figure 33. The bar diagram depicts the pooled mean ± SEM values (n = 6) for the maximal tension evoked by PE (1 μmol/L) alone and the maximal tension generated by the addition of glycine (3.0 mmol/L) in PE (1 μmol/L) constricted state, in the absence as well as presence of MK-801(10 μmol/L) in the aortic rings of SHR strain.

*$p < 0.05$ compared to respective control.

$^{††}p < 0.01$ compared to glycine treatment without MK-801.

CHAPTER 5. DISCUSSION

5.1. Hypotensive effect of glycine in normotensive rats

Acute administration of glycine (1 mmol/kg), a precursor/metabolite of L-serine, evoked a fall in MAP in normotensive WKY rats. Glycine acts as a coagonist in the NMDA receptors (Kasai et al., 1978). NMDA receptors are predominant in the central nervous system and their activation evokes cerebral vasodilatation (Faraci et al., 1993). There are studies showing the release of NO in central nervous system during the activation of NMDA receptors (Lin et al., 1999; Nagata et al., 1995). However, studies have already reported the existence of NMDA receptors in the periphery (Crespi et al., 2000; Slomowitz et al., 2002; Deng et al., 2002). Thus, using two NMDA antagonists the present study provides pharmacological evidence of NMDA receptor involvement in glycine evoked changes in BP. This suggests the presence of vascular NMDA receptors in the normotensive WKY rats. Glycine is transported into cells through selective amino acid transporter (Zafra et al., 1989; Hashimoto. 2006). Sarcosine, N-methyl glycine, at a dose of 100 mg/kg is known to inhibit the transport of glycine into cells and thus enhance the extracellular concentration of glycine (Yang et al., 2010). When extra cellular glycine level is enhanced by blocking glycine transporter, there is significant concentration of glycine available to interact with a limited population of all surface NMDA receptor present on selective renal vascular beds (Deng et el., 2002). Thus pretreatment with sarcosine inhibits transport of glycine and increases the availability of glycine to act on cell surface NMDA receptors to elicit a higher fall in MAP compared to treatment with glycine alone in normotensive WKY rats.

These findings confirm that the glycine mediated fall in MAP is in fact mediated via activation of vascular NMDA receptors. Recently, it has been suggested that glycine evoked fall in MAP was significantly attenuated in the presence of NOS inhibitor, L-NAME (100 μmol/kg), in normotensive WKY rats (Mishra et al., 2008b). Taking these results together, the glycine evoked fall in MAP is due to activation of endothelial NMDA receptor which is linked to NO-mediated vasodilatation subsequent to Ca^{2+} induced NOS activation.

5.2. Hypertensive effect of glycine in hypertensive rat models

Both in L-NAME pretreated hypertensive WKY rats and SHR, paradoxically, glycine evoked increases in MAP, an effect that is opposite to what was observed in normotensive WKY rats. Pretreatment with NMDA antagonist, MK-801 (75 mg/kg), completely abolished the BP elevating effect of glycine in both hypertensive rat models. Again, pretreatment with memantine (50 mg/kg), another NMDA antagonist, also abolished the hypertensive effect of glycine. These results suggest NMDA receptors mediate the BP elevating effect of glycine following acute glycine administration. Glycine evoked elevation in MAP encountered in chronic L-NAME treated WKY and SHR strains were exaggerated further when the responses to glycine was determined following pretreatment with glycine transporter inhibitor, sarcosine.

5.3. Comparison of glycine and L-serine effect in the regulation of BP

It is important to discuss the changes in hemodynamic parameters following acute glycine and L-serine administration in the regulation of BP, since glycine serves as a

precursor/metabolite to L-serine (Greenstein et al., 1961). We have recently reported that L-serine, a non-essential amino acid derived from glycine promotes concentration and endothelium-dependent vasodilatation in rat mesenteric arterioles (Mishra et al., 2008a). In rat models, L-serine evokes a reversible, dose-dependent fall in MAP following acute intravenous administration without increasing HR; interestingly, both *in vitro* and *in vivo* effects of L-serine were more pronounced in NO compromised state (Jalil, 2008; Mishra et al., 2008a; Mishra et al., 2008b). The antihypertensive effect of L-serine likely involves the activation of endothelial calcium activated potassium (K_{Ca}) channels (Jalil. 2008: Mishra et al., 2008a; Mishra et al., 2008b). We have also demonstrated that L-serine evoked fall in BP in rat models is primarily due to decrease in splanchnic vascular resistance which subsequently increases blood flow to splanchnic vascular beds (Mishra et al., 2010). Glycine, a precursor and metabolite of L-serine, also evoked a fall in BP in normotensive rats. Unlike serine, glycine administration exerts a pressor response in hypertensive rats. Both the depressor and pressor responses evoked by glycine are sensitive to blockade by NMDA antagonists (Mishra et al., 2008b). L-serine mediated vasodilator and hypotensive effect was abolished in the combined presence of apamin and charybdotoxin but not NMDA antagonist. In contrast, glycine mediated changes in BP responses remain unaffected in the combined presence of apamin and charybdotoxin but the responses were abolished by NMDA antagonist (Mishra et al., 2008b). L-serine although derived from or metabolized to glycine, the vascular and BP responses to L-serine and glycine are governed by different mechanisms and are not interlinked. It has already been reported that glycine is an inhibitory neurotransmitter and mediates its effect primarily by activating its receptors. Glycine receptors are prevalent in brain and spinal

cord. Activation of these receptors enhances chloride flux and subsequent hyperpolarization of cells (Imboden et al., 2001 and Lynch, 2004). Glycine also acts as a co-agonist at NMDA glutaminergic receptors along with glutamate (Kasai et al., 1978 and 1980). The existence of peripheral NMDA receptors in the kidney and heart has been previously demonstrated (Leung, 2002). Some reports suggest that acute glycine infusion increases blood flow and enhanced perfusion in the heart and kidney, via activation of NMDA receptors in these organs (Deng et al., 2002; Leung 2002). Data from the present thesis supports this notion and is consistent with the above findings. Glycine decreases MAP by reducing vascular resistance perhaps by increasing blood flow to heart, kidney and brain. This is primarily due to activation of NMDA receptors present in these organs since inclusion of NMDA receptor antagonists MK-801 as well as memantine inhibited these responses. This result confirms that the glycine evoked depressor response is mediated through activation of NMDA receptor and is consistent with the previous studies that reported glycine can act as a coagonist at NMDA receptor (Kasai et al., 1978). In hypertensive rats, glycine administration increases MAP by increasing vascular resistance in several organs that includes kidney, brain and heart by activating NMDA receptors since NMDA antagonists, MK-801, memantine attenuated the exaggerated pressor response to glycine noted in hypertensive rats. These two opposite responses to glycine suggest that in normotensive WKY rats, glycine activates endothelial as well as renal NMDA receptors that in turn increase intracellular Ca^{2+}. This activates Ca^{2+} dependent NOS and subsequently results in increased NO production. Increase in NO activates the cGMP pathway that is likely responsible for vasodilatation in kidney, heart and brain vessels which reflects the fall in systemic vascular resistance (↓ TPR) and the

subsequent fall in MAP. In chronic L-NAME pretreated WKY rats, NOS is inhibited; therefore, glycine mediated activation of endothelial NMDA receptors does not contribute to increase NO generation. Activation of vascular NMDA receptor increases calcium influx which in turn increases contractility and contributes to increase in BP. In SHR, acute glycine administration also increases MAP by activating vascular NMDA receptors. It is well documented that in SHR strain NOS availability is diminished due to progressive endothelial dysfunction which contributes to arterial stiffness and loss of elasticity (Marque et al., 1999). Thus, both the depressor and pressor responses to glycine noted in normotensive and hypertensive rat models are mediated via activation of endothelial/vascular NMDA receptors respectively.

5.4. Glycine induced changes in systemic and regional hemodynamic parameters

In normotensive WKY rats**,** acute glycine administration evoked a fall in MAP whereas in hypertensive L-NAME treated WKY rats and SHR, it evoked an increase in MAP. In WKY rats, acute administration of glycine decreased TPR and increased CO whereas in hypertensive rats, TPR increased and CO was decreased following glycine administration. In WKY rats acute glycine administration, increased blood flow to a significant extent to heart, kidney and brain whereas in hypertensive rats, blood flow was lower in heart, kidney, brain, pancreas, skeletal muscle and diaphragm. Changes in blood flow to the splanchnic region following glycine administration did not show any statistical significance in both normotensive and hypertensive rat models. Changes in vascular resistance in fact, drives the changes in blood flow. Pooled data from several experiments demonstrated that following glycine administration, peripheral organ

vascular resistance significantly decreases in heart, kidney and brain with subsequent increase in blood flow to these vital organs. This contributes to the fall in MAP in normotesive WKY rats. Conversely, glycine administration increases organ vascular resistance in heart, kidney, brain, pancreas, skeletal muscle and diaphragm in SHR and L-NAME treated hypertensive WKY rats. This increase in organ vascular resistance in hypertensive rat models contribute to decrease in blood flow to these organs/tissues and results in an increase in TPR and MAP. Vascular resistance and blood flow are inversely proportional to each other and changes in these parameters contribute to the changes in blood flow and the accompanying changes in BP (Brunton et al., 2005). Again, acute administration of the NOS inhibitor, L-NAME, inhibits glycine mediated vasodilator and hypotensive responses in normotensive rats. On the contrary in hypertensive rat models, NOS is compromised. Therefore, glycine evokes vasoconstrictor and hypertensive responses. This study clearly explains the differential effects of glycine in normotensive and hypertensive rat models and both the effects are mediated by endothelial/vascular NMDA receptors.

5.4.1. Glycine targets vital organs in rat models

Results of the present study demonstrate that unlike L-serine, glycine targets all the vital organs such as heart, kidneys, brain, pancreas, diaphragm and skeletal muscle in hypertensive rats. In normotensive rats glycine primarily targets heart, kidney and brain. Compared to glycine, L-serine targets predominantly the splanchnic vascular beds and decreases the splanchnic vacular resistance which contributes to the increase in

splanchnic blood flow and the subsequent decrease in TPR with reduction in BP in both normotensive and hypertensive rat models (Mishra et al., 2010).

5.5. Proposed mechanism for glycine mediated vasodilatation

It is likely that NMDA receptors are expressed on EC and VSMC. The level of its expression could vary in different regional vascular beds. Accordingly, in aortic rings isolated from normotensive WKY rats, glycine via activation of NMDA receptor present on EC, promotes elevated $[Ca^{2+}]_i$ and eNOS activity that results in NO-dependent vasodilatation. However, in NO compromised state such as in SHR strain, that exhibits endothelial dysfunction, NMDA receptor mediated vasodilatation is minimal or absent. Under such conditions, addition of glycine promotes NMDA-receptor mediated vasoconstriction via finite population of NMDA receptors present on VSMC. This may account for the vasoconstrictor responses to glycine observed in aortic rings isolated from SHR strain. This is also consistent with the elevated VSM tone seen under endothelium-denuded states in normotensive WKY rats. Thus, glycine mediated increase in basal tone is due to the activation of a limited population of vascular NMDA receptors. In contrast, when experiments were performed to examine the effects of glycine in PE constricted vessels, it evoked concentration-dependent vasodilatation and this effect of glycine was abolished when L-NAME was present in the organ bath. Thus, these data confirm that in aortic rings with intact endothelium, glycine promotes vasodilatation via releasing NO. Since inclusion of NOS inhibitor, L-NAME, inhibited glycine-mediated vasodilatation, activation of endothelial NMDA receptor is linked to NO mediated vasodilatation. It is reported that in the brain, NMDA receptor activation promotes NO production via

activation of nNOS and results in cerebral vasodilatation (Garthwaite et al., 1989; East et al., 1991; Faraci et al., 1993). It has also been reported that NMDA receptor functions as a membrane Ca^{2+} channel since activation of this receptor increases Ca^{2+} influx (Davis et al., 2003). Stimulation of Ca^{2+}/Calmodulin dependent protein kinase signaling cascade by the activated NMDA receptor has been shown to regulate NO dependent axon and neurite outgrowth (Wayman et al., 2004). Although NMDA receptors are prevalent in the central nervous system and brain stem, their existance has also been reported in the peripheral vasculature (Crespi et al., 2000, Deng et al., 2002, Leung et al., 2002). Glycine is a co-agonist at NMDA glutaminergic receptor (Kasai et al., 1978 and 1980; Danysz et al., 1998). Glycine evokes its peripheral effect by binding to the vascular NMDA receptors and results in an increase in Ca^{2+} influx. This elevated Ca^{2+} probably activates eNOS present in the EC of the blood vessels. Activation of NOS produces NO in EC which diffuses to adjacent VSMC and causes cGMP dependent vasodilatation in normotensive rats. Our results from *in vitro* studies demonstrate that in endothelium denuded aortic ring preparartion glycine failed to evoke vasodilatation. Also in hypertensive rat models, glycine evokes a pressor response compared to depressor responses observed in normotensive rats. These responses are sensitive to NMDA antagonist. Results from our studies suggest that in the absence of endothelium, following denudation or in the presence of L-NAME, acute addition of glycine could activate only vascular NMDA receptors present on VSMC, elevate cytosolic free Ca^{2+} to enhance vascular tone. This results in increase in systemic vascular resistance and elevation in BP. Thus, our *in vitro* results are consistent with our *in vivo* observations. Acute glycine administration evoked a fall in BP in normotensive WKY rats with intact endothelium. Whereas, in L-NAME

pretreated hypertensive WKY rats, where endothelial function was compromised by inbition of NOS, glycine produced a pressor response via activation of NMDA receptors present on the VSMC. NO-dependent vasodilatation is limited or negligible in the established phase of hypertension in SHR strain (14 week old) due to progressive endothelial dysfunction (Zhou et al., 2008). In SHR strain, acute glycine administration increases BP via activation of NMDA receptors present on VSMC since NO availability is compromised due to endothelial dysfunction. A proposed mechanism that governs glycine evoked vasodilatation and fall in BP in normotensive rats and glycine evoked vasoconstriction and increase in BP in hypertensive rats are schematically reperesented (Figure 34).

5.6. Proposed mechanism for glycine mediated vasoconstriction

In SHR strain, there is endothelial dysfunction and NMDA receptor activation following glycine administration, could not promote increased NO generation. Studies have already shown the existence of NMDA receptors in large conduit vessel such as aorta (Crespi et al., 2000). Thus, it is possible that in NO compromised state, glycine mediated activation of vacular NMDA receptor promotes intracellular Ca^{2+} influx and results in increased vascular tone. These *in vitro* results are consistent with our *in vivo* results where we have already demonstrated that acute glycine administration increases BP both in chronic L-NAME tereated WKY and SHR strains. Thus, acute single dose glycine administration activates endothelial NMDA receptors leading to enhance NO generation subsequent to Ca^{2+} dependent NOS activation and vasodilatation in blood vessels of critical organs such as kidneys, heart and brain. This contributes to a

significant fall in systemic vascular resistance and fall in MAP in normotensive rats. While a small population of NMDA receptor present on VSMC promotes increases in VSM basal tone. The dominant NO-mediated vasodilatation buffers enhanced vascular tone and causes fall in TPR and MAP. In contrast, in chronic L-NAME treated rats and SHR strains enhanced vascular tone in the absence of NO generation (in L-NAME model) or compromised NO function (SHR model), induced by single administration of glycine predominates and contributes to its pressor response in hypertensive rat models. Previously, it was reported that glycine intake has potential to reduce BP by reducing oxidative stress and it is useful in normalization of blood glucose level (Martinez-Abundis et al., 2003; Hafidi et al., 2006; Katayama et al., 2007). Glycine intake is suggested to be beneficial in the management of CNS related disorders (Buchanan et al., 2007). However, our results suggest that acute glycine intake may be not beneficial in hypertensive subjects as suggested before since its acute administration is shown to evoke vasoconstriction and elevation of BP. In hypertensive rats, we demonstrate the possible mechanisms that contribute to the pressor response (Figure 34).

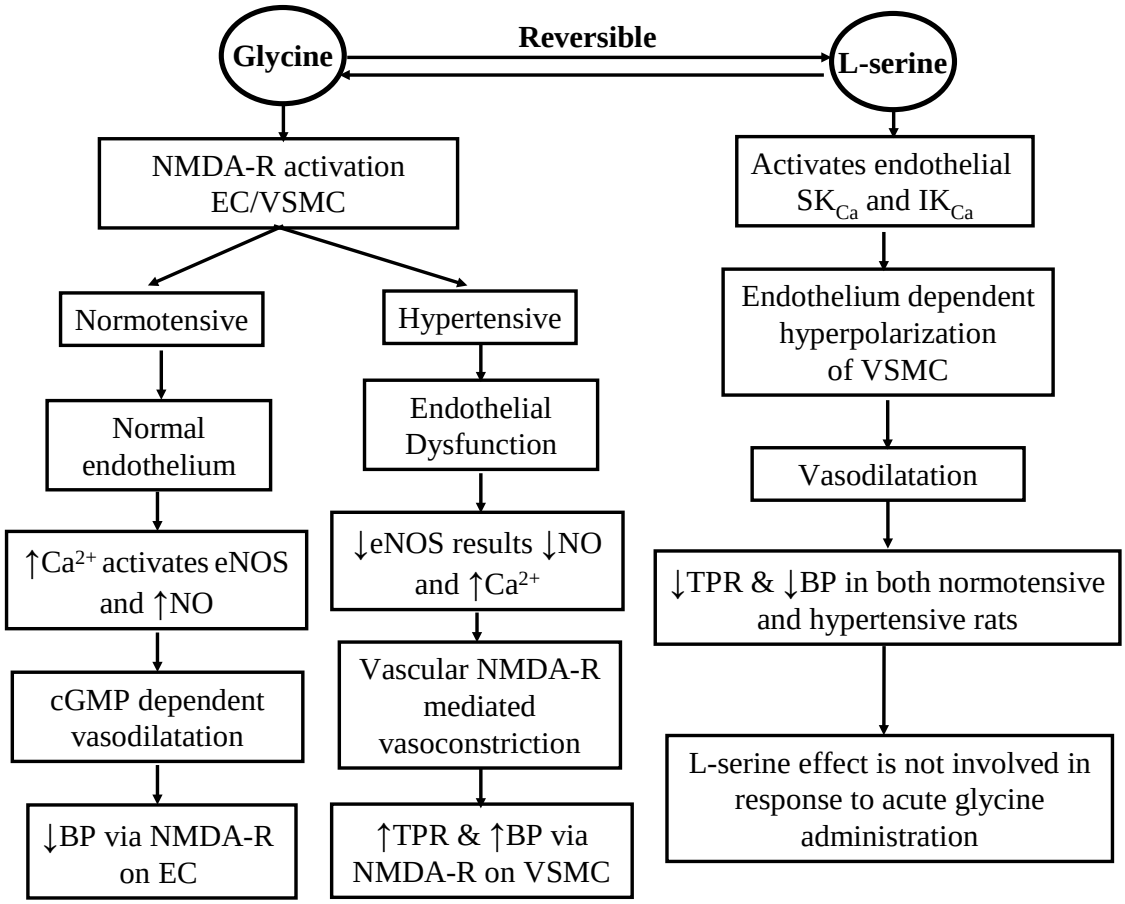

Figure 34. This schematic diagram compares the proposed mechanism of vasodilatation/fall in BP in normotensive rats and vasoconstriction/elevation in BP evoked by glycine in hypertensive rats. L-serine evokes only vasodilatation and fall in BP in all rat models. Note: Acute glycine responses shown in the present study is not mediated by its interconversion to L-serine. Glycine effects are mediated solely via activation of NMDA-receptors present on EC/VSMC.

VSMC, vascular smooth muscle cell; EC, endothelial cell; NMDA, N-methyl D-aspartate; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; cGMP, cyclic 3', 5' guanosine monophosphate; BP, blood pressure; SKca, calcium activated small conductance potassium channel; IK_{Ca}, calcium activated intermediate conductance potassium channel.

CHAPTER 6. CONCLUSIONS AND FUTURE WORK

6.1. Summary of the present study

Glycine is not essential to diet since it can be formed in the body from other amino acids namely L-serine, L-alanine and L-threonine. Glycine is synthesized in liver of most vertebrates by glycine cleavage system which is also called glycine synthase and serine hydroxyl methyltransferase. This reaction is reversible and essential for cellular homeostasis. This reversible reaction of glycine to L-serine or L-serine to glycine is to maintain intracellular one carbon group which is essential for thymidine synthesis. Glycine and L-serine also plays an important role in purine and pyrimidine synthesis which are component of nucleotide base pairs. Other than that purines are part of ATP, GTP, cAMP biomolecules. Thus both glycine and L-serine are considered as conditional essential amino acids that are important for cellular growth and its survival. Glycine is well studied and considered as an inhibitory neurotransmitter in central nervous system. It also acts as a co-agonist at NMDA receptor along with D-serine. Glycine and its peripheral effect in vasculature are not well studied. So, this study focuses on the acute peripheral vascular effects of glycine. Although glycine and L-serine serves as a precursor/metabolite to each other, their mechanism of action is entirely different in normotensive and hypertensive rat models. Both Glycine and L-serine evoke their responses in an endothelium dependent manner. However, glycine mediated vasodilator effect is dependent on NO and endothelial NMDA receptor activation. NMDA receptor activation increases intracellular calcium which activates eNOS and contributes to NO synthesis. NO diffuses to adjacent VSMC and evokes vasodilatation via cGMP and PKG

mediated effects on VSMC. In contrast, L-serine mediated vasodilator responses are rather mediated via activation of endothelial SK_{Ca} and IK_{Ca} channels present on the endothelium. Activation of SK_{Ca} and IK_{Ca} channels hyperpolarizes adjacent VSMC. Thus, in normotensive rat models both glycine and L-serine evoke vasodilator responses and the mechanisms that contribute to the final response (vasodilatation/fall in BP) are entirely different. In contrast, L-serine evokes vasodilatation and a fall in BP in hypertensive rats but acute administration of glycine enhances vasoconstriction and elevates BP further in these rats. In hypertensive rat models endothelium-mediated/NO-dependent vasodilator effect following endothelial NMDA receptor activation is significantly blunted due to endothelial dysfunction. Under such conditions, the vascular NMDA receptor (present on VSMC) mediated Ca^{2+} dependent vasoconstrictor effect predominants to elevate BP in hypertensive rat models. Glycine in hypertensive rat models evokes a contractile response since it activates NMDA receptor and increases intracellular calcium which increases vascular tone in absence of NO and subsequently contributes to increase in MAP. In summary the acute vascular response to glycine are not mediated by its bioconversion to L-serine. This information is new and provides useful insight for future studies.

Thus, the results of this study demonstrate that glycine mediated responses are in fact due to activation of vascular NMDA receptors. The present study provides adequate new information to propose a long-term chronic study of glycine administration to ensure the beneficial effect of glycine that was reported earlier. Although a report suggested long term glycine intake induces formation of nitrosating agent in presence of NO, this

nitrosated compound may pose risk of gastrointestinal cancers (Cupid et al., 2004). Finally and most importantly we conclude that glycine mediated depressor/pressor responses are linked to NO generation or lack of NO in normotensive vs hypertensive states. Moreover, glycine administration affects blood flow to the critical organs such as, kidney, heart and brain. The present study also suggests that there are NMDA receptors could be expressed in vasculature and they may show differential responses between normotensive and hypertensive rats. This may assume significance following food intake with elevated plasma levels of glycine. So glycine could play an important role in cardiovascular disease states.

6.2. Significance of the study

Following administration of acute bolus dose of L-serine or its precursor/metabolite glycine is accompanied by fall in BP in normotensive rats but glycine increases whereas L-serine decreases BP in hypertensive rat models (Mishra et al., 2008b). The present study demonstrates that glycine induced depressor and pressor response is mediated by vascular/endothelial NMDA receptor. Unlike L-serine, which targets splanchnic and small intestinal vascular beds, glycine a nonpolar amino acid, predominantly targets brain, heart and kidney but not the splanchnic vascular beds. Results of this study, infact, opens new avenues to study more precisely the beneficial effect of glycine intake in NO compromised state as several studies have suggested such possibilitis (Martinez-Abundis et al., 2003, Hafidi et al., 2006, Katayama et al., 2007 and Buchanan et al., 2007). It is possible that in long term glycine intake or treatement, glycine could be converted to L-serine. So the responses to glycine what we observed

following acute administration in the hypertensive rat models, could be different. But in hypertensive rat models, NO availability is still compromised and we know from the present *in vitro*, *in vivo* and regional hemodynamic studies that glycine effects are mediated following activation of vascular/endothelial NMDA receptors. In conclusion, it is to be noted that glycine induced changes in BP involve different vascular beds with completely different mechanisms compared to its metabolite/precursor L-serine.

6.3. Future Work

i. Long term chronic studies with glycine and L-serine administration are required to demonstrate whether over a period of time glycine is converted to L-serine. Their likely contribution to BP regulation following chronic treatment is currently unknown. It has to be addressed by studies in conscious rats using radiotelemetry methodology.

ii. A detailed plan and robust studies to continue the acute *in vitro* effects of glycine on renal vascular beds and isolated renal arterioles obtained from normotensive and hypertensive rats are warranted since changes in maximal blood flow and vascular resistance occurred in this vascular bed.

iii. It is also worth to study glycine responses in isolated coronary and cerebral blood vessels isolated from normotensive and hypertensive rats.

iv. The demonstration of NMDA receptors either by receptor binding studies or glycine mediated changes in $[Ca^{2+}]_i$ level in smooth muscle cells and rat aortic endothelial cells will add another new dimension.

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